2018

# FRACTIONATION OF LIGNIN DERIVED COMPOUNDS FROM THERMOCHEMICALLY PROCESSED LIGNIN TOWARDS ANTIMICROBIAL PROPERTIES

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Digital Object Identifier: https://doi.org/10.13023/ETD.2018.130

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THESIS

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A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science in Biosystems and Agricultural
Engineering in the College of Engineering at the University of Kentucky

By:

Luke Dodge

Lexington, Kentucky

Director: Dr. Jian Shi, Assistant Professor of Biosystems and Agricultural Engineering Department

Lexington, Kentucky

2018

ABSTRACT OF THESIS

# FRACTIONATION OF LIGNIN DERIVED COMPOUNDS FROM THERMOCHEMICALLY PROCESSED LIGNIN TOWARDS ANTIMICROBIAL PROPERTIES

The overuse of antibiotics in agriculture is an emerging concern, due to their potential detrimental impact to the environment. This study focuses on exploring antimicrobial properties of lignin derived compounds. Lignin is of interest as a feedstock to replacing some petroleum-based chemicals and products because it is the most abundant source of renewable aromatic compounds on the planet. Two lignin rich streams, residues from the enzymatic hydrolysis of dilute acid and alkaline pretreated corn stover, were decomposed *via* pyrolysis and hydrogenolysis, respectively. The resulting liquid oils were subjected to sequential extractions using a series of solvents with different polarities. Chemical compositions of the extracted fractions were characterized through HPLC and GC/MS. These extracted compounds were screened against *Saccharomyces cerevisiae* (*S. cerevisiae*), *Escherichia coli*, and *Lactobacillus amylovorus* for antimicrobial properties. Six lignin model monomers: guaiacol, vanillin, vanillic acid, syringaldehyde, 2,6-dimethoxyphenol, and syringic acid were compared to the oils and extracted fractions for antimicrobial properties. Development of lignin-derived chemicals with antimicrobial properties could provide a novel use for this underutilized natural resource.

KEYWORDS: lignin, pyrolysis, hydrogenolysis, liquid-liquid extraction, antimicrobial

Luke Dodge

April 26, 2018

# FRACTIONATION OF LIGNIN DERIVED COMPOUNDS FROM THERMOCHEMICALLY PROCESSED LIGNIN TOWARDS ANTIMICROBIAL PROPERTIES

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Acknowledgments

I am extremely grateful for the people who helped me throughout my research. I first want to thank my graduate advisor Dr. Jian Shi for his support through my graduate career at the University of Kentucky. I would like to extend a thank you to my other committee members Drs. Sue Nokes and Barbara Knutson for their insightful comments and guidance on my research project. I would like to thank Dr. Xiaowen Chen at NREL for providing the lignin samples and Dr. Lalitendu Das for helping with the GC/MS and GPC analysis. I also like to thank Dr. Dorin Boldor and Dr. Pranjali Muley from LSU for their helping on with the pyrolysis.

I want to thank my mom and dad for all they have done to help me succeed during my graduate career. Your love and endless support was the motivation I needed to help me through this process.

I would like to acknowledge the National Science Foundation under Cooperative Agreement No. 1355438 and 1632854 and the National Institute of Food and Agriculture, U.S. Department of Agriculture, Hatch-Multistate project under accession number 1003563 for supporting this research.

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## CHAPTER 1: INTRODUCTION

### Introduction

The goal of this chapter is to review previous studies to establish what research has been accomplished in the past and where the gap in research lies. This literature review focused primarily on the topics of lignocellulosic biomass, lignin, thermochemical lignin decomposition, sequential extraction, lignin derived antimicrobials, and their potential applications in a biorefinery. The objectives, research approach, and research questions were also laid out in this chapter.

### 1.1 Literature Review

*1.1.2 Lignin*

Lignin, which accounts for 15-25% of a plant biomass, is one of the major components, apart from cellulose and hemicelluloses, in the lignocellulosic matrix. Lignin is a three dimensional amorphous polymer consisting of methoxylated phenylpropane units. Lignin plays biological roles essential to the life of vascular plants. Lignin is responsible for the plants' rigid structure and water transport due to its hydrophobic nature (Biology 2017). Despite extensive studies on lignin its exact form is unknown. It is believed that lignin is formed by the polymerization of three major monomers: *p*-coumaryl (H), coniferyl (G), and sinapyl (S) alcohols. Different plants have different ratios of these three monolignols. For example, rye straw has an G:S:H ratio of 43:53:1 whereas rice straw has a ratio of 45:40:15 (Sun, Fang et al. 2000). In recent

years, the idea that lignin could have antimicrobial properties has emerged as a research interest. There are several other potential applications of lignin: including but not limited to, bio-dispersants, epoxy resins for circuit boards, adhesives, wood panel products, and cement additives (Ten and Vermerris 2015).

**Table 1:** Predominant linkages (Santos et al., 2013; Chakar and ragauskas, 2004)

| Linkage Type | Dimer Structure | % linkages in soft wood | % linkages in hard wood |
|---|---|---|---|
| β-*O*-4 | Phenylpropane β-aryl ether | 45-50 | 60 |
| 5-5 | Biphenyl and dibenzodioxocin | 18-25 | 20-25 |
| β-5 | Phenylcoumaran | 9-12 | 6 |
| β-1 | 1,2-Diaryl propane | 7-10 | 7 |
| α-*O*-4 | Phenylpropane α-aryl ether | 6-8 | 7 |
| 4-*O*-5 | Diaryl ether | 4-8 | 7 |
| β-β | β-β-Linked structures | 3 | 3 |

As the most abundant source of renewable aromatic compounds on the planet, lignin is gaining interest as a feedstock in replacing petroleum-based chemicals and products. It is however an under-utilized natural resource due to its structural heterogeneities (Zhao, Simmons et al. 2016). The challenge is that the 5-5 and β-5 carbon bonds are difficult to cleave and constitute a significant portion of the lignin's linkages (Table 1). Unless scientists can determine a way to use the whole lignocellulose feedstock including lignin, the cellulosic biofuel industry will remain stagnant (Zeng, Zhao et al. 2014).

*1.1.3 Thermochemical Lignin Decomposition*

In its raw form, lignin does not have many uses. Currently, most of the lignin is burned to produce heat and power. The breakdown of lignin helps to access desirable functions that are not achievable when lignin is a polymer. Once lignin polymers are

broken down, the low molecule compounds become suitable for upgrading to fuel and chemicals. Several lignin depolymerization pathways are being developed, including pyrolysis, catalytic oxidation, catalytic transfer hydrogenolysis, ionic liquid-based catalysis, and biological depolymerization. Pyrolysis and catalytic transfer hydrogenolysis (CTH) are the two thermochemical breakdown methods that will be discussed here.

Pyrolysis is the breakdown of large molecules into smaller ones by the application of heat in the absence of oxygen. During pyrolysis, lignin is heated to temperatures between 160-900°C where cleavage of the ether (C-O) and C-C linkages takes place (Yang, Yan et al. 2007). Lignin pyrolysis produces a range of pyrolytic aromatic compounds in oil form in addition to gas products and residual char. The yield and composition of pyrolytic oil are influenced by many factors, including lignin type and operation conditions (Mullen, Boateng et al. 2010).

Research has shown that CTH is an attractive alternative to traditional hydrogenation. With CTH using an alcohol as a liquid hydrogen donor, as compared to gaseous hydrogen in traditional hydrogenation, CTH is safer. During CTH of lignin, hydrogen-donating solvents, such as formic acid, methanol, ethanol, isopropyl alcohol (IPA), and tetralin etc., release hydrogen molecules at elevated temperatures usually with help of a catalysts. The hydrogen is transferred in-situ for hydrogenation reactions between the lignin bonds, causing them to breakdown and thus leading to lignin depolymerization (Toledano, Serrano et al. 2013). IPA remains popular choice due to its relative low cost and easy subsequent separation from the reaction mixture (Kim, Simmons et al. 2017).

*1.1.4 Sequential Extraction*

Lignin depolymerization products are usually a mixture of aromatic compounds. In order to find the best use of these compounds, it is necessary to investigate a separation method that is cost effective and efficient in recovering specific aromatic compounds. Several separation techniques can be applied, including chromatography, evaporation, and filtration, etc. These methods present their own challenges: time, effectiveness, cost, and recyclability after the extraction. Liquid-liquid extraction (LLE), commonly known as solvent extraction and partitioning, is a method to separate compounds based on their relative solubilities in two different immiscible liquids. The two liquid phases usually have different polarities, so the compounds partition into two phases depending on the polarities of the molecules.

LLE has been applied to fractionate bio-oil recovered from pyrolysis of lignocellulosic biomass (Ren, Ye et al. 2017). In such a process, a series of organic solvents were used to extract groups of lignin derived compounds. Results show the molecular weight of the recovered fraction decreased and the total phenolic and methoxyl concentrations increased after LLE (An, Wang et al. 2017). The authors also conducted a DPPH (2, 2- diphenyl-1-picryl-hydrazyl-hydrate) assay to determine a positive or negative antimicrobial effect. With the ability to separate out different groups of lignin derived compounds, the possibility of better understanding how certain organically derived lignin compounds can have antioxidant properties can be examined and more precise testing for antioxidant properties can be achieved. A research group at the University of Tennessee in 2017 looked at how to optimize the sequential extraction process using organic solvents. Optimal ratios of organic solvent to water mixtures were

established in order to ensure that one step of the sequential extraction did not extract everything (Ren, Ye et al. 2017). It should be noted that the optimal order for the organic solvents is least polar to most polar. Once separated, the isolated groups of compounds can then be used for different applications based on the properties exhibited by the specific groups. This study done by Tennessee tested switchgrass pyrolysis. However, it is not clear whether the same protocol can be used for other biomass feedstocks, such as corn stover. Nor is it clear if the same protocol can be used with bio-oil derived from other lignin depolymerization methods such as CTH.

### *1.1.5 Lignin Derived Antimicrobials*

A study was published in 1979 about the antimicrobial properties of several lignin derived compounds (Zemek, Košíková et al. 1979). In this study they looked at how certain compounds, such as eugenol, isoeugenol, syringaldehyde, ferulic acid, etc. affected the growth of certain microbes such as *Saccharomyces cerevisiae, Candida albicans, Escherichia coli, Bacillus licheniformis, and Aspergilus niger.* This study found that the side chains on these compounds played a significant role in antimicrobial activity. In contrast, it was noted that groups with oxygen (-OH, -CO, -COOH) in the side chains were less effective. Results from the earlier literature demonstrated antimicrobial properties of lignin; however, more mechanistic understanding is needed before a transition from using petroleum based antibiotics to lignin derived ones can occur.

First, the antimicrobial effects of lignin derivatives on microorganisms must be determined. It is thought that some simple organic compounds, such as phenols, exhibit ionophoric properties; which allows the transportation of particular ions across the cell membrane either through a carrier or a channel (Tsukube, Yamashita et al. 1991).

Exposing certain microorganisms to ionophoric compounds may disrupt cell membrane causing leakage or complete cell lysis. It is hypothesized that lignin derived compounds could possibly function in a similar way, as lignin can be broken down to produce a variety of phenolic compounds. This is an area that is not fully understood and additional research still needs to be conducted to understand the extent of phenolic compounds that can be produced from lignin (Fache, Boutevin et al. 2015).

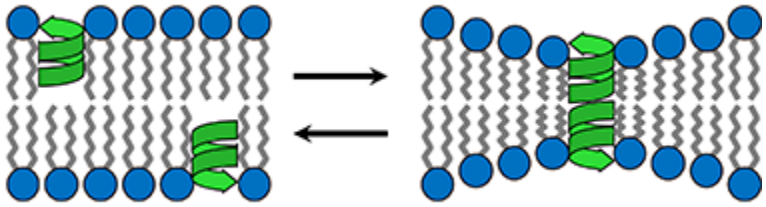


**Figure 1:** Carrier and channel ionophores (David and Rajasekaran 2015).

In order to better utilize lignin's antimicrobial activity, it is essential to explore the selectivity against different microorganisms. Cell wall structure and cell wall composition are one of the main factors differentiating one microorganism from another (Figure 1). A gram-negative bacterial cell wall contains a thin layer of peptidoglycan in its periplasmic space between the two lipid membranes, the inner and outer. The leaflets on the outer membrane contain lipopolysaccharides and facilitate non-vesicle-mediated transport through channels. Gram-positive bacteria, mycobacteria, and fungi all lack the presence of an outer membrane and have a thin cell wall. Gram-positive bacteria are made up of a single lipid membrane surrounded by a cell wall composed of a thick layer of peptidoglycan and lipoteichoic acid. Glycolipids and porins are also found in gram-positive cell walls which are anchored to the cell membrane by diacylglycerol. This is

important because it has been hypothesized that ionophore resistance relies on extracellular polysaccharides which can bar ionophores from the cell membrane (Russell and Houlihan 2003). Fungi contain a single plasma membrane surrounded by a cell wall. This cell wall is made up of various layers of the polysaccharides chitin, β-glucan and mannan (Brown, Wolf et al. 2015).

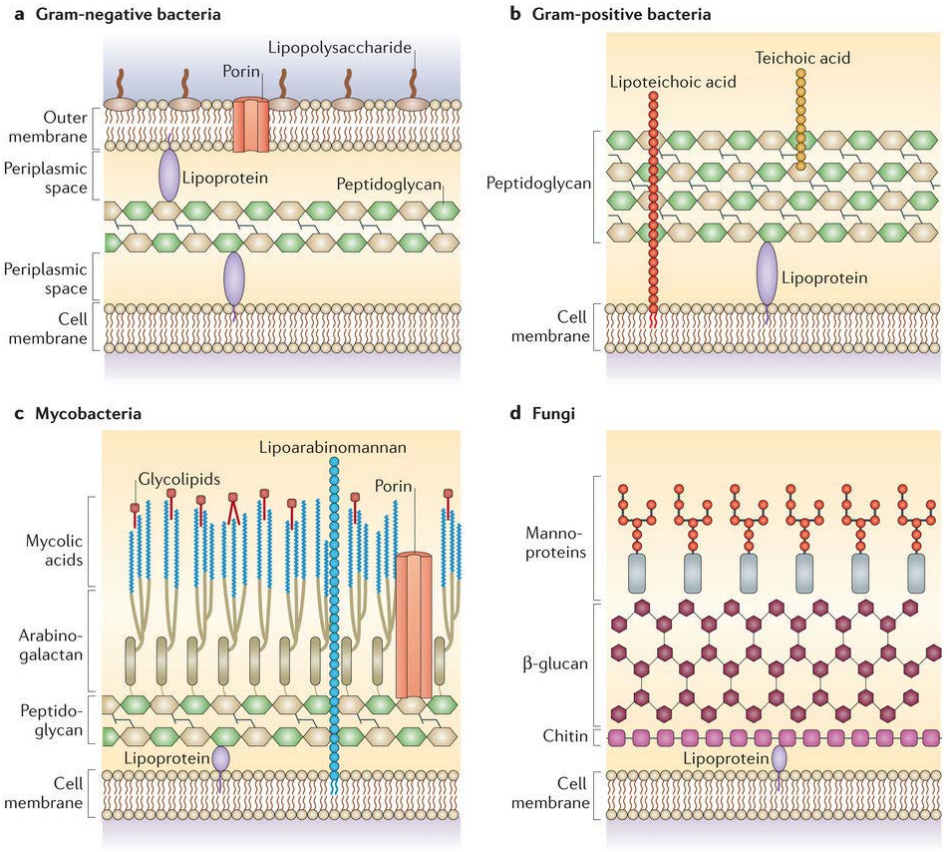



**Figure 2:** Illustrations of the cell wall of **a)** Gram-negative bacteria, **b)** Gram-positive bacteria, **c)** mycobacteria, and **d)** fungi. Adapted from (Brown et al., 2015)

*E.coli* is gram negative and *L. amylovorus* is gram positive. This means that these two bacteria should behave differently when subjected to antimicrobial compounds due to their difference in cell wall structure and composition. Table 2 shows some of the major differences between gram positive and gram negative microorganisms.

**Table 2:** Comparison of gram positive and gram negative bacteria (Diffen 2014, Brown, Wolf et al. 2015)

| | **Gram Positive** | **Gram Negative** |
|---|---|---|
| **Gram Reaction** | Retain crystal violet dye and stain dark purple. Retain stain when washed with alcohol or water. | Can be decolorized to accept counter stain, stain ted or pink. Does not retain stain when washed with alcohol or acetone. |
| **Peptidoglycan Layer** | thick multilayered | thin single layer |
| **Periplasmic Space** | absent | present |
| **Outer Membrane** | absent | present |
| **Flagellar Structure** | 2 rings | 4 rings |
| **Resistance To Physical Disruption** | high | low |
| **Cell Wall Composition** | 100-120 Å (angstrom) thick<br>lipid content is low<br>murein content is high | 70-120 Å thick<br>lipid content is high<br>murein content is low |

*S. cerevisiae* is the fungus that is widely used in fermentation, and thus, it is important to also understand how single-celled fungi are different from bacteria. To start, fungi are eukaryotes while bacteria are prokaryotes. Bacteria are single celled while fungi are multicellular, with *S. cerevisiae* being an exception. The shape of fungi varies from one to another, while bacteria have three different shapes. Lastly, fungi reproduce both sexually and asexually, where bacteria reproduce sexually via binary fission (Golden

2011). Hopefully by understanding the differences between fungi, gram-negative, and gram-positive bacteria, it will help understand whether and why certain lignin derived compounds have a selective inhibition properties.

### *1.1.6 Applications in a Biorefinery*

There are many potential applications to which lignin derived compounds can be applied, but this study is positioned on the potential use of lignin compounds to improve ethanol fermentations. The ethanol formation process is vulnerable to microbial contamination. *L. amylovorus* contamination at a minimum can lower the efficiency of an ethanol fermentation process and at its worst, it can lead to no alcohol production. Some of the *E. coli* species are health-harmful varieties rather than spoilage microorganisms, like lactic acid bacteria. *L. amylovorus* is a dominant contaminant because this bacteria is well adapted to survive under low pH, low oxygen, and under high ethanol concentrations (Beckner, Ivey et al. 2011). *L. amylovorus* inhibits *S. cerevisiae* in two main ways: it competes for the same nutrients that *S. cerevisiae* needs, and/or it produces lactic and acetic acids that shift in the pH to an uncomfortable zone for yeast. Currently, penicillin is commonly used to control contamination by *L. amylovorus* (Bayrock, Thomas et al. 2003). A study done in 2003 found that using 2,475 U/l of penicillin increased the *S. cerevisiae* growth by two fold and allowed for an increase in ethanol production. They also looked at pulsed and continuous addition of the penicillin and found no major difference (Bayrock, Thomas et al. 2003). Although penicillin helps the ethanol production process, it is not a sustainable solution. The overuse of antibiotics in agriculture, including biorefineries, is an emerging concern due to their potential detrimental impact to the environment and ecosystems.

If an antimicrobial product can be formed from lignin and applied to the ethanol fermentation, then the corn or cellulosic ethanol production process would become more environmental friendly and profitable. Lignin taking up roughly 20% of the lignocellulosic biomass. Turning a portion of lignin into antimicrobials that are biodegradagle can be advantageous since the footprint of antibiotics will be reduced in Dried Distillers Grains and Solids (DDGS). With a big concern being how many antibiotics both humans and animals consume, the ability of these antibiotics to degrade naturally would prevent them from continuing to build up in the environment.

It is hypothesized that some of the lignin derived compounds have selective inhibition properties that will allow *S. cerevisiae* to grow and prevent contamination by other microbes. One potential advantage of lignin-based antimicrobials is their biocompatibility because the antimicrobial compound would degrade in the environment just like lignin does naturally. As an added bonus, the leftover grain from the fermentation would not contain harmful compounds, besides lignin, and thus, making the DDGS safe for animal consumption and the antibiotics that pass through cattle would degrade in the environment.

### 1.2 Research Motivations

The overarching research goal is to explore the antimicrobial activity of lignin-derived molecules as a means of finding a potential application in the biofuel industry; such as replacing the antibiotics currently applied to ethanol fermentation. If a renewable resource, like lignin, can be used to make chemicals that have similar effects to current antibiotics, then the spent grains would be safe for animals and would be more biocompatible in the natural environment. However, there is a gap in research about

whether it is possible to turn lignin into antimicrobial compounds and whether these compounds have selectivity on microorganisms of interest.

## 1.3 Objectives

This study focuses on antimicrobial properties within lignin depolymerization products derived from two types of lignin streams. Two thermochemical processes will be used to depolymerize the lignin streams and the oil will be sequentially extracted using a prescribed sequence of solvents. These extracted fractions and commercial monolignols will be screened against *Saccharomyces cerevisiae* (*S. cerevisiae*), *Escherichia coli* (*E. coli*), and *Lactobacillus amylovorus* (*L. amylovorus*). The specific objectives are to:

1) Identify major lignin degradation compounds in the byproduct of two different thermochemical treatment methods: pyrolysis and catalytic transfer hydrogenolysis (Chapter 2)
2) Identify fractions of lignin degradation compounds sequentially extracted from pyrolysis and catalytic transfer hydrogenolysis oil by a prescribed sequence of organic solvents (hexane, petroleum ether, chloroform, and ethyl acetate). (Chapter 2)
3) Screen commercial mono-lignols, guaiacol, vanillin, vanillic acid, syringaldehyde, 2,6-dimethoxyphenol, syringic acid, and sequentially extracted lignin fractions for microbial inhibitory properties (Chapter 3).

## 1.4 Research Approach and Research Questions

### *1.4.1 Research Approach:*

Lignin was extracted from corn stover pretreated by two different methods, i.e., dilute acid (DA) and alkaline (AL), representing two common technologies used in

today's cellulosic biorefineries. The pretreatment was followed by enzymatic hydrolysis to remove fermentable sugars. The remaining lignin-rich materials will be further decomposed using pyrolysis and catalytic transfer hydrogenolysis (CTH). These different compound mixtures will be analyzed by gas chromatography – mass spectrometry (GC-MS) to determine what percentage of different lignin compounds are present in each. Sequential extraction will be used to fractionate the lignin compounds based on the solvent's polarity.

*1.4.2 Research Questions*:

The goal is to answer the following questions through this research project.

Q1: How do the lignin oils differ when the starting lignin-rich residue (dilute acid vs. alkaline pretreated, enzymatically hydrolyzed) after undergoing depolymerization through pyrolysis and CTH?

Q2: Can compounds be extracted from lignin oil derived from pyrolysis and CTH using solvents of different polarity to generate a range of different fractions of lignin derived compounds?

Q3: What is the tolerance of *S. cerevisiae*, *E. coli*, and *L. amylovorus* to lignin monomers and lignin oil derived fractions?

# CHAPTER 2: FRACTIONATION AND CHARACTERIZATION OF LIGNIN DERIVED COMPOUNDS FROM THERMOCHEMICALLY PROCESSED LIGNIN

## Introduction

A composition analysis was conducted to establish the percentage breakdown of lignin, glucose, and xylose in the two different samples. FTIR was run to help confirm the composition analysis results. The DA and AL samples were broken down using pyrolysis and CTH. These resulting oils were then subjected to a sequential extractions using four solvents with different polarities: hexane, petroleum ether, chloroform, and ethyl acetate. The chemical compositions of the extracted fractions were characterized using GC/MS. GPC was used to examine the size distribution of untreated and processed lignin samples and the results demonstrated the effectiveness of the two breakdown methods on lignin depolymerization.

## 2.1. Experimental

### *2.1.1 Materials*

The two lignin samples were provided by National Renewable Energy Laboratory (NREL) with more details provided in section 2.1.2. The organic solvents: hexane, petroleum ether, chloroform, ethyl acetate, isopropyl alcohol, and palladium activated charcoal were purchased from Sigma-Aldrich.

### *2.1.2 Lignin Sample Preparation*

Corn stover were pretreated by dilute acid (DA) and alkaline (AL) at NREL. The DA pretreatment was conducted at 175°C, 30g $H_2SO_4$/kg of dry biomass, 30% solids loading, and residence time of 8 minutes. The AL pretreatment used 0.1g NaOH/ g

biomass with 15% solid loading at 80°C for two hours. The pretreated biomass was hydrolyzed by NREL with cellulase enzymes and the solid residues retained as the lignin streams for this study. Enzymatic hydrolysis following both pretreatments was conducted at 15% solids loading, 64 mg CTec2/g dry biomass HTec2 was loaded with 8:1 ratio (Chen, Kuhn et al. 2016). The two lignin samples were denoted as "DA" and "AL" lignin as shorthand notation for the lignin rich residues collected after dilute acid and alkaline pretreated corn stover followed by enzymatic hydrolysis. Upon receiving the DA and AL lignin samples, they were washed with DI water and dried at 105°C overnight and then ground using a mortar and pestle.

*2.1.3 Pyrolysis Protocol*

Pyrolysis of DA and AL lignin samples was performed at Dr. Boldor's lab at Louisiana State University (LSU). Approximately 7.5g of each pretreated sample was added directly to a custom pipe reactor (SS-316, 20" length, 3/8" I.D., 1/2" O.D.). The biomass was distributed over the 8.0" length of the reactor. Pieces of cotton were loaded into both ends of the reactor to keep the biomass within the reactor. The reactor was then placed inside a horizontal induction coil (6 turns, 9" length, 2.0" I.D.) such that the biomass inside the reactor was positioned within the coil. One end of the reactor was attached to a $N_2$ source by way of high-temp tubing and the flow rate was regulated by a MC-50SLPM-D Mass Flow Controller (Alicat Scientific, Tucson, Arizona). The other end of the reactor was attached to cold trap collection system using high-temperature plastic tubing (~2.5", 0.2" I.D.). The tubing was attached to a 1/8" steel tube that travelled through the interior and ended at the bottom of a collection vial. The cold trap collection system consisted of this collection vial placed inside a larger beaker containing

both dry ice and acetone. Gaseous products produced by the pyrolysis of the biomass entered the cold trap system through the 1/8” pipe connected to the reactor.

Prior to each pyrolysis experiment, $N_2$ gas flowed through the custom-designed reactor at a rate of 0.45 L/min for a minimum of 20 minutes in order to purge the system of any excess $O_2$. When the purging session was completed, the $N_2$ flow rate was adjusted to 0.15 L/min and the induction heater was turned on. The PID portion of the IR2 Supermeter was programmed to the desired set point temperature (500 °C) and was configured to send voltage changes to the HFI model induction heater (RDO Induction L.L.C., Washington, New Jersey) in order to increase or decrease the power of the induction heater such that the reactor would be stabilized at 500 °C. The experiment ran for 50 minutes during which time gaseous products condensed into the cold trap system. The masses collected before and after the experiment and were used to determine the liquid yield percent.

*2.1.4 Catalytic Transfer-Hydrogenolysis (CTH) Protocol*

CTH was run using a Parr Reactor (Moline, IL, Series 4560 Mini Reactor) at a set temperature of 270±5 °C for 1 hour. The CTH was preheated for 45 minutes to reach the set temperature. The solvent, isopropyl alcohol (IPA), was used at 15.7g and 2g of sample was added to the reactor. The catalyst, palladium on activated charcoal (Pd/C), was used at 10% of the sample concentration (0.2g) (Kim, Simmons et al. 2017). After the reaction was finished, air was used to quickly cool the reactor to 100°C, then ice was used to cool the reactor to 25°C. The contents in the reactor was then recovered by rinsing with acetone. The collected liquid and suspended solids was separated by

centrifuging at 4000 rpm for 10 minutes and the liquid part was dried in a vacuum oven at room temperature for 48 hours to remove the acetone and IPA.

*2.1.5 Gas Chromatography-Mass Spectrometry (GC/MS) Protocol*

Dichloromethane (1ml) was added to re-suspend the lignin derived oil from pyrolysis and CTH. This oil/ dichloromethane suspension was filtered using a 0.2 micron syringe filter and then injected into the GC/MS for analysis. A 70 minute, GC/MS method was adapted to identify the lignin depolymerization products from the CTH and pyrolysis reactions. The analysis was performed on Agilent 7890B GC coupled 5977B MS (Santa Clara, CA) with a HP-5MS (60 m × 0.32 mm) capillary column. The temperature program started at 40 °C with a holding time of 6 minutes and increased to 240 °C at 4 °C $minutes^{-1}$ with a holding time of 7 minutes; then the temperature was raised to 280 °C at 20 °C $minutes^{-1}$ with a holding time of 8 minutes. Helium was used as a carrier gas with a flow rate of 1.2 mL $minutes^{-1}$. Peaks were identified using the MS library of compounds. Compounds with the highest probability that were also derivatives of lignin were selected. The area of each peak was compared to the total peak area of a total sample, to determine the percentages of each compound. The detection limitation of MS detector is 800 g/mol. Compounds with a molecular weight larger than 800 g/mol were unable to be identified however, they can be tested using GPC analysis.

*2.1.6 Gel Permeation Chromatography (GPC) Protocol*

The weight-average molecular weight ($M_w$) and the number-average molecular weight ($M_n$) of the raw, processed, and residue lignin samples were determined using GPC (McClelland, Motagamwala et al. 2017). An Ultimate3000 HPLC system equipped with an Ultra Violet (UV) detector was used. Separation was accomplished in a mobile

phase of tetrahydrofuran (THF) at a flow rate of 0.5 ml minutes$^{-1}$, using a Mixed-D PLgel column (5 μm particle size, 300 mm x 7.5 mm i.d., linear molecular weight range of 200 to 400,000 u, Polymer Laboratories, Amherst, MA) at 50°C. Elution profiles of materials eluting from the column were calibrated using low molecular weight polystyrene standards (Product No. 48937, Sigma-Aldrich) a at UV absorbance of 280 nm. Mw is the weight average molecular weight. Mn is the number average molecular weight. Polydispersity Index (PDI) was calculated using the equation: PDI= Mw/Mn (McClelland, Motagamwala et al.).

*2.1.7 Sequential Extraction Protocol*

In order to better determine the lignin derived compounds produced, a sequential extraction was performed to separate the compounds based on polarity. These groups would then be tested to see which compounds have an inhibitory effect on different microbes. A water-isopropanol mixture (80:20 v/v) and four solvents with different polarities were used: from most polar to least polar, water, ethyl acetate, chloroform, petroleum ether, and hexane (Miller 1998). Normal value in Table 3 were calculated by dividing the solvent polarity by water's polarity.

**Table 3:** Organic Solvents Polarity (Harris 2015)

| Solvent | Solvent Polarity | Normal Value |
|---|---|---|
| Hexane | 0.1 | 0.009 |
| Petroleum Ether | 0.1 | 0.009 |
| Chloroform | 2.7 | 0.265 |
| Ethyl Acetate | 4.4 | 0.431 |
| Water | 10.2 | 1.000 |

Each of these solvents has a different extraction efficiency for different compounds based on the solvents polarity. For example, chloroform has a high extraction efficiency for furans, phenolics, and ketones. Ethyl acetate, on the other hand, has a high extraction efficiency for organic acids. A study by Ren et al. in 2017 found the optimal order to use these solvents for lignin compound extraction was water, hexane, petroleum ether, chloroform, and ethyl acetate (Ren, Ye et al. 2017). The flow chart for this extraction protocol is shown below in Figure 3.

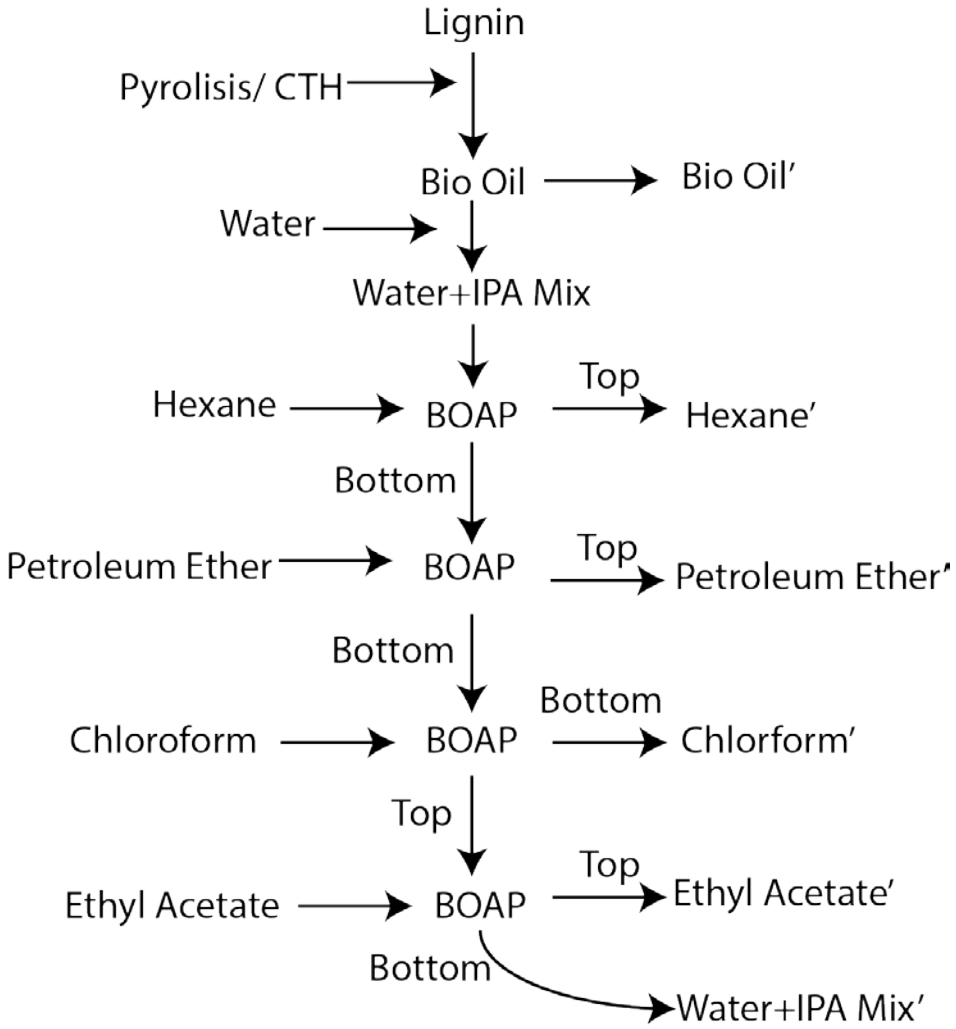



**Figure 3:** Sequential extraction flow chart (BOAP stands for bio-oil aqueous phase)

*2.1.8 Fourier-transform Infrared Spectroscopy (FTIR)*

FT-IR spectra were generated using a FTIR spectrometer (Waltham, MA, Thermo-Nicolet Nexus 670 FTIR). All spectra were generated over accumulative 64 scans with a resolution of 4cm$^{-1}$ in the range of 700-4000 cm$^{-1}$. A spectrum was generated without any sample (blank) to account for background noise.

*2.1.9 Statistical Analysis*

All experiments were conducted in duplicates or triplicates and the data are presented as means and standard deviations. The statistical analysis, ANOVA and two-

ways Tukey's test, was performed by SAS® 9.4 (SAS Institute, Cary, NC, US), with a significance level of $P<0.05$ for all the data obtained from experiments.

## 2.2. Results and Discussion

### *2.2.1 Composition Analysis on the Raw Lignin Samples*

A composition analysis was conducted to establish the makeup of the DA and AL lignin samples prior to thermochemical depolymerization. The results are shown in Table 4. This composition analysis of the two lignin starting materials will also help explain why the oil yields from pyrolysis and catalytic transfer hydrogenolysis were lower than the typical yield from Kraft lignin. The DA lignin sample had a lignin content of 62.83% as compared to the AL lignin sample at 58.91% (having a P-value of 0.45). The glucan content for the DA and AL lignin samples were 27.54% and 18.63% respectively with a P-value of 0.02. The xylan content for the DA and AL lignin samples were 6.04% and 7.58% respectively with a P-value of 0.09. A composition analysis published in 2007 on raw corn stover found a glucan content of roughly 36% (Öhgren, Bura et al. 2007). It is common that the biomass derived lignin contains large portion of glucan and xylan due to the incomplete enzymatic hydrolysis (Chen, Guo et al. 2009). The carbohydrate impurity in the DA and AL lignin samples could explain why the lignin oil yields were found to be lower in this study than reported with pure Kraft lignin. However, any further purification step on the lignin samples would add extra cost in chemicals and energy. Thus in this study, the received lignin samples were used directly because they represent the real lignin samples one could recover from a biorefinery. In order to gain more insights about the composition and chemistry, FTIR was run on the lignin samples and results correlated to composition analysis.

**Table 4:** Composition of DA and AL pretreated lignin samples

| | Lignin | Glucan | Xylan | Total | Unknown |
|---|---|---|---|---|---|
| **DA Lignin** | 62.83 ± 1.81 | 27.54 ± 0.24 | 6.04± 0.02 | 96.41 ± 2.07 | 3.60 ± 2.07 |
| **AL Lignin** | 58.91 ± 5.67 | 18.63 ± 1.68 | 7.58 ± 0.71 | 85.12 ± 8.06 | 14.88 ± 8.06 |

*2.2.2 FTIR Spectra of Raw Lignin Samples*

The chemical and structural changes in NREL DA and AL lignin samples as compared to Kraft lignin were examined by FTIR. The FTIR spectra shows that the lignin received from the NREL was very different from Kraft lignin (figure 4). All three lignin samples display an absorption band at 3,400 $cm^{-1}$, which represents aliphatic and aromatic O-H groups (Faix 1991). The band at 2,930 and 2,840 $cm^{-1}$ can be designated with the vibrations of C-H from the $CH_2$ and $CH_3$ groups (Cachet, Camy et al. 2014).The C=C of aromatic skeletal vibrations were imitated by the peaks at 1,595 and 1,510 $cm^{-1}$ (Prado, Erdocia et al. 2016). These peaks were significantly lower in intensity with the DA and AL lignin as compared to the Kraft lignin. The bands associated with 1,460 and 1,420 $cm^{-1}$ can be accredited to the C-H deformations in $CH_2$ and $CH_3$ groups and C-H aromatic ring. A significant decreases in peak intensity at 1,420 $cm^{-1}$ for DA and AL lignin was noticed when compared to the Kraft lignin, demonstrating possible breakdown of the $CH_2$ and $CH_3$ groups. The bands linked to guaiacyl (G) and syringyl (S) lignin units were detected at 1,220 and 1,110 $cm^{-1}$ (García, Erdocia et al. 2012, Gordobil, Moriana et al. 2016). The band at 1,220 $cm^{-1}$ is akin to C-C, C-O, and C=O stretching (G) showing decrease in intensity when DA and AL lignin was compared to Kraft lignin (U.S. Department of Energy 2016). The band at 1,110 $cm^{-1}$ assigned to aromatic C-H in plane deformation (S) from DA and AL lignin also showing a decrease in intensity when compared to Kraft lignin. The peak at 1,050 $cm^{-1}$ refers to C-O vibrations of the

crystalline cellulose region. With DA and AL lignin having a main peak at 1,050 $cm^{-1}$ this indicates that both of these lignin samples have a higher cellulose content than the Kraft lignin (Li, Knierim et al. 2010). These FTIR results are in agreement with the composition analysis results that were obtained from the DA and AL lignin streams. Both DA and AL lignin sources were much less pure than the Kraft lignin. The higher impurity is probably contributing to the decrease in oil yield from the thermochemical depolymerization of these two lignin streams.

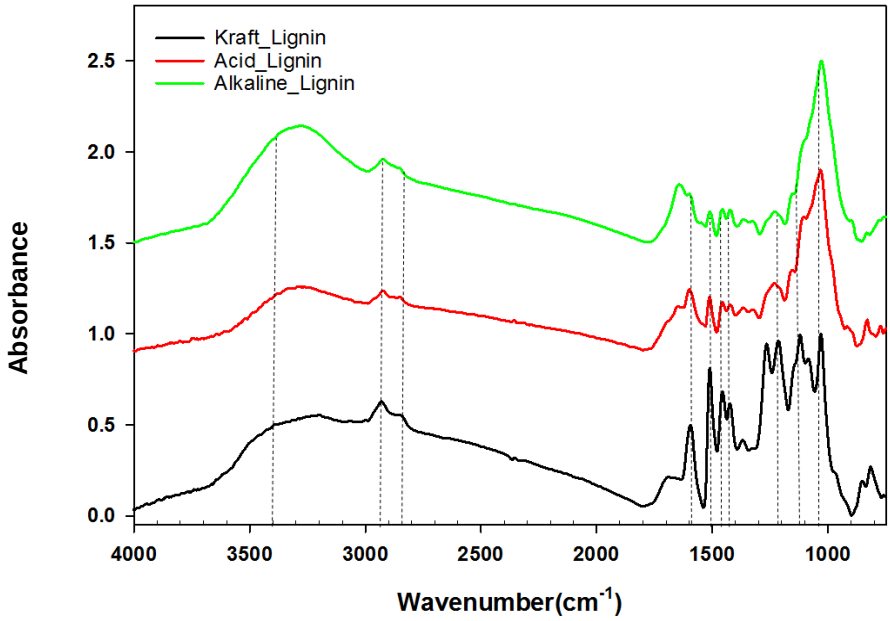



**Figure 4:** FTIR spectra of DA and AL lignin as compared to a commercial Kraft lignin

*2.2.3 Products Distribution after Pyrolysis and CTH of DA and AL Lignin*

A mass balance was attempted to determine where DA and AL lignin ended up after the pyrolysis and CTH reaction. Table 5 shows the oil, solids, and gas percentages from the mass balance of the lignin streams. Pyrolysis produced more lignin oil in both DA and AL lignin streams than the CTH did. DA lignin pyrolysis produce a higher percentage of lignin oil as compared to the CTH with 15.34% and 8.27% respectively.

This same trend can be observed in the AL lignin. With the pyrolysis producing a higher percentage of lignin oil as the CTH with 20.76% and 9.92% respectively. Only the oil percentages of this mass balance should be considered because solids from pyrolysis were not collected. Also, no gas for either set of sample was collected. Therefore, it cannot be determined how much solids were lost due to vessel transfer and user error.

Figure 5 shows only the oil yields and their standard deviations. It is thought that because these lignin samples have a relative large sugar content that a lower oil yield is observed. A statistical analysis was performed on the lignin oil samples. An ANOVA was performed and displayed that one of the samples exhibited a statistical difference. With the ANOVA, AL CTH lignin had a P-value of 0.04. A T-test was then performed to establish which combination of pretreatment and thermochemical breakdown process was statistically different. A comparison was run within and between treatments. It was found that the DA CTH and DA Pyrolysis was the only statistical different with respective P-values of 0.03.

The results from this statistical analysis can be found in Appendices I. A study completed in 2007 which explored pyrolysis reactions for a variety of different feedstocks found an oil yield of 22-55% depending on the feedstock. They also found that the lower the ash content in a sample, the more oil yield they were able to collect (Fahmi, Bridgwater et al. 2008). In both lignin streams, pyrolysis produced a larger percentage of lignin oil than CTH, but only DA pyrolysis was the only statistical different. Within treatments, neither CTH nor pyrolysis was statistical different.

**Table 5:** Oil, solid, and gas products from pyrolysis and CTH of DA and AL lignin

| | Pyrolysis | | | CTH | | |
|---|---|---|---|---|---|---|
| | % oil | % solid | % gas | % oil | % solid | % gas |
| DA lignin | 15.34 ± 0.96 | - | - | 8.27 ± 1.48 | 37.28 ± 0.53 | 54.46 ± 2.02 |
| AL lignin | 20.76 ± 5.67 | - | - | 9.92 ± 1.34 | 44.22 ± 1.51 | 45.87 ± 0.17 |

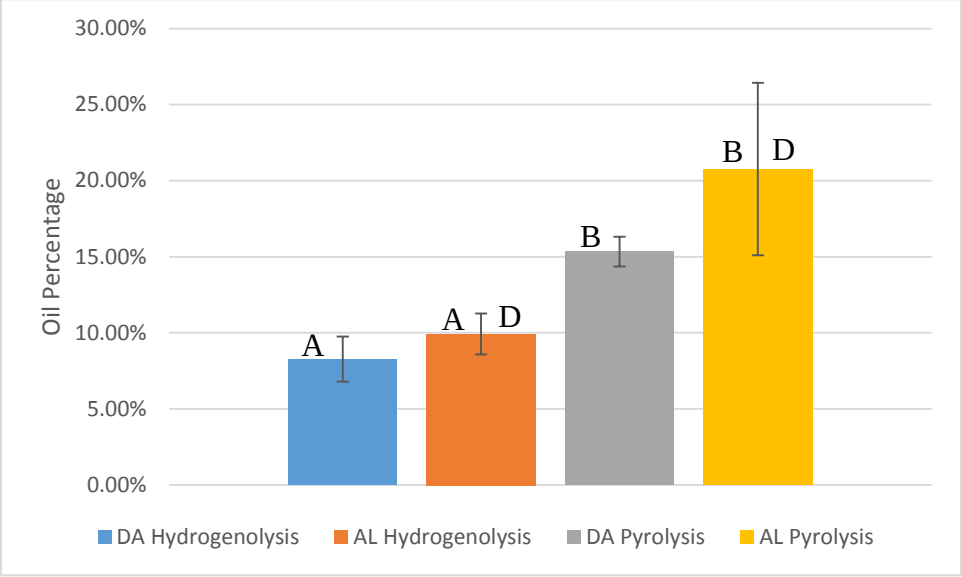



**Figure 5:** CTH and pyrolysis bio oil yields for Da and AL lignin

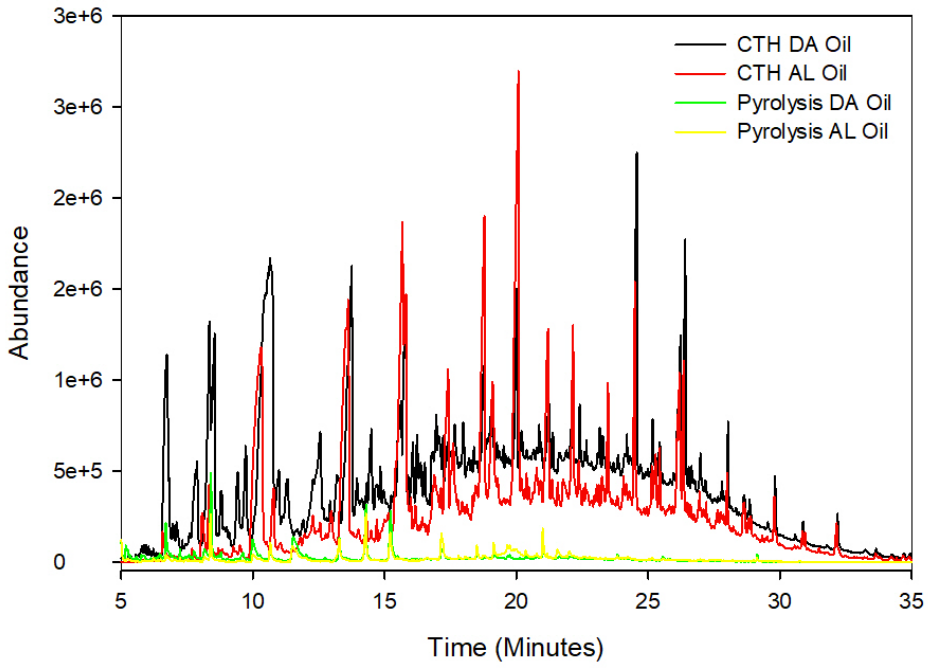



**Figure 6:** GC/MS chromatography of liquid lignin oils

A GC/MS was run on the different lignin oils. These results are shown in Figure 6. One key distinction here is that different methods do not produce different compounds, but the ratios of compounds produced is different for different methods. All peaks are relative and only the percentages of the peaks can be compared. Comparing the CTH DA and the CTH AL peak for 4-ethyl-phenol, it is observed from Table 7 and Appendices A that the DA's peak represents 20% of the total products while AL has a peak of only 12%. Comparing the pyrolysis DA and AL peak for 4-ethyl-2met-phenol, it was shown that the DA has a 6% concentration while the AL has a 14%. A study published in 2011 ran pyrolysis on raw corn stover and found four major monomeric phenolic compounds produced: phenol, 4-vinyl phenol, 2-methoxy-4-vinyl phenol, and 2,6-dimethoxy phenol, with yields ranging from 1-4% (Patwardhan, Brown et al. 2011). Patwardhan et al. 2011 also found that the total yield of phenolic compounds was 18 wt%. Results from our study indicate that it is possible to tune the thermochemical depolymerization process to

produce desirable compounds; in other words, a few steps can be taken to depolymerize lignin to produce a higher concentration of the desired compound. This is achieved by different combinations of pretreatment and thermochemical break down process. Both lignin streams can produce a variety of compounds, and there are some differences in the amount of compound produced based on the type of lignin samples and the breakdown process that was performed.

*2.2.4 Sequential Extraction and GC/MS Characterization of Extracted Fractions*

In order to see the compounds in the lignin oil separated, a sequential extraction was performed. The mass fractions of the lignin oil separated by each solvent extracts are presented in Table 6. Most lignin compounds were extracted into the less polar solvent. Hexane has a relative polarity of 0.009 which is less polar when compared to water at 1 (Miller 1998). For CTH of both DA and AL lignin, the sequential extraction extracted a lower percentage of compounds further down the process; with the exception for chloroform. The same trend was seen for sequential extraction of pyrolytic lignin oils from DA and AL lignin. Chloroform for all sequential extractions extracts a higher percentage of oil than the previous step. This is most likely due to chloroform having a relative polarity of 0.259 compared to petroleum ether with a relative polarity of 0.009 (Miller 1998). Both hexane and petroleum ether have relative polarities that are roughly the same. The water + IPA mix for CTH DA and CTH AL extracted a relatively low percentage, <5%, and thus, it can be said that roughly all, >95%, of the stating lignin derived compounds for CTH were extracted into a more non-polar environment than water + IPA Mix. The pyrolysis extraction had less of the oils extracted into hexane than the CTH oil. With both pyrolysis DA and pyrolysis AL having 20% in the water + IPA

mix stage, it can be observed that roughly 80% of the pyrolysis starting compounds were extracted with the solvents. There is a difference in the polarity preference between the two break down methods: with CTH preferring a more non-polar environment than pyrolysis. Meaning that CTH allowed more compounds to be sequentially extracted. This is not surprising given that with CTH these is the addition of hydrogen and thus more hydrophobic compounds are produced in CTH than compared to pyrolysis.

**Table 6:** Mass fractions from different organic phases during sequential extractions

| Sequential Extraction Breakdown | | | | |
|---|---|---|---|---|
| | CTH DA Lignin | CTH AL Lignin | Pyro DA Lignin | Pyro AL Lignin |
| Hexane' | 57.66% | 29.17% | 23.68% | 22.78% |
| Petroleum Ether' | 10.80% | 9.29% | 19.48% | 13.19% |
| Chloroform' | 19.89% | 46.15% | 27.97% | 29.21% |
| Ethyl Acetate' | 10.80% | 10.58% | 10.37% | 15.56% |
| Water + IPA Mix' | 0.85% | 4.81% | 18.50% | 19.26% |

An extraction fractionation GC/MS is shown in Figure 6. Hexane extracted lignin oil showed peaks in line with the lignin oil at earlier elution times while chloroform extracted lignin oil has peaks in line with later elution times. The ethyl acetate and water + IPA mix phase had no identifiable compounds.

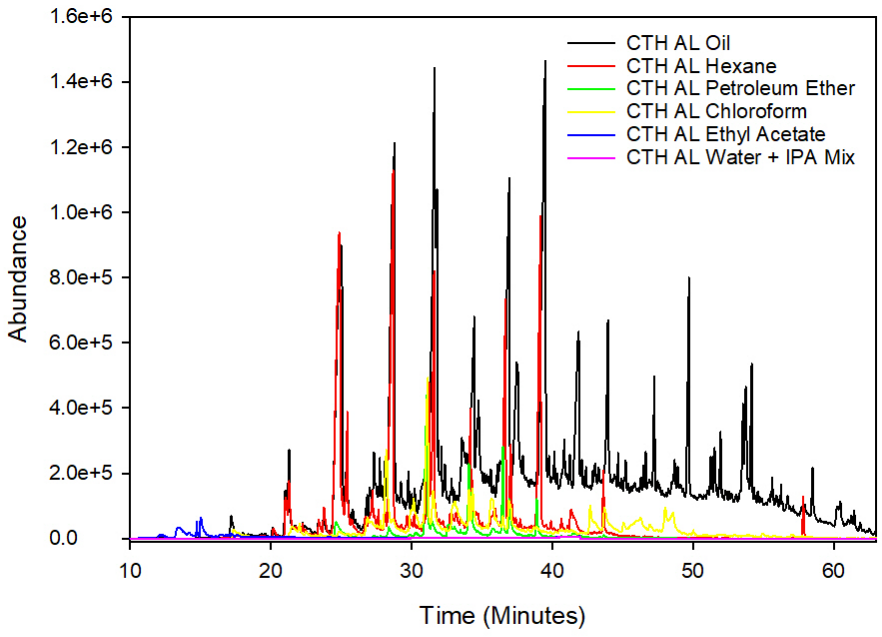



**Figure 7:** GC/MS chromatography of extracted fractions from CTH AL lignin oil

Table 7 shows the percentage results of the GC/MS compounds. Ethyl Acetate and the Water + IPA mix phases were left out as no compounds were identified in either. The compounds listed in Table 7 are the compounds that were able to be identified and quantified by the GC/MS. The start lignin oil is made up of many different compounds but, it has a few phenols that are present in high concentrations: 4-ethyl-phenol (12%), 4-ethyl-2-methoxy-phenol (8%), and 2,6-dimethoxy-phenol (13%). As the sequential extraction takes place, only specific compounds were pulled out at each step and because of this their relative concentrations increased. It is speculated that some of the oil's main compounds persist in both hexane and petroleum ether because the solute have reached their saturation point. Further calculations and/or research are required to establish the solubility of these lignin compounds. Petroleum Ether has a very similar polarity to hexane, which could explain why these lignin derived compounds, not picked up by hexane, want to move into the petroleum ether. Chloroform is observed to extract

different compounds from hexane and petroleum ether. This is mostly likely due to the increase in polarity. With chloroform having a relative polarity of 0.259 it still attracts non-polar compounds but is attracting compounds that are slightly more polar than the previous steps. Performing a sequential extraction is beneficial when it is performed using solvents in order of increasing polarity.

**Table 7**: Percentage of compounds in fractions sequentially extracted from CTH of AL lignin

| Time (Minutes) | Compound Name | Bio Oil' | Hexane' | Petroleum Ether' | Chloroform' |
|---|---|---|---|---|---|
| 10.5 | Phenol, 4-ethyl- | 12.04% | 23.68% | 9.53% | |
| 13.8 | Phenol, 4-ethyl-2-methoxy- | 7.74% | 18.90% | 3.80% | |
| 16.0 | Phenol, 2,6-dimethoxy | 13.15% | 5.85% | 33.23% | 31.47% |
| 17.6 | Phenol, 4-methoxy-3-(methoxymethyl)- | 3.63% | 3.60% | 11.03% | |
| 19 | Benzene, 1,2,3-trimet | 6.82% | | | |
| 19.5 | 5-Sec-butylpyrogallol | 4.48% | | | |
| 20.3 | 3,5-Dimethoxy-4-hydro | 12.52% | | | |
| 25.4 | Creosol | | 4.54% | | |
| 21.2 | Phenol, 2-methoxy- | | 2.29% | | |
| 31.6 | Phenol, 2-methoxy-4-propyl- | | 8.78% | | |
| 36.7 | Benzoic acid, 3,4-dimethoxy- | | 8.58% | | |
| 37 | 4-Ethoxy-3-methoxybenzyl alcohol | | 2.91% | | |
| 39.2 | Benzeneacetic acid, 4-hydroxy-3,5-dimethoxy- | | 12.92% | | |
| 32.6 | 4-Ethylcatechol | | | 2.32% | |
| 34.2 | Benzoic acid, 4-hydroxy-3-methoxy- | | | 8.56% | 17.33% |
| 35.8 | Phenol, 5-methoxy-2,3-dimethyl- | | | 2.80% | |
| 36.5 | Benzene, 1,2,3-trimethoxy-5-methyl- | | | 12.86% | |
| 36.9 | 3-Ethoxy-4-methoxybenzyl alcohol | | | 9.74% | |
| 38.9 | Benzeneacetic acid, 4-hydroxy-3,5-dimethoxy- | | | 6.12% | |
| 28.2 | 1,2-Benzenediol, 3-methoxy- | | | | 22.21% |
| 29.9 | 1,2-Benzenediol, 4-methyl- | | | | 4.53% |
| 31.5 | Phenol, 3,4-dimethoxy- | | | | 14.39% |
| 37 | 2-Propanone, 1-(4-hydroxy-3-methoxyphenyl)- | | | | 4.01% |
| 42.7 | Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)- | | | | 6.07% |
| | ***>2% concentrations*** | ***60.39%*** | ***92.04%*** | ***100.00%*** | ***100.00%*** |

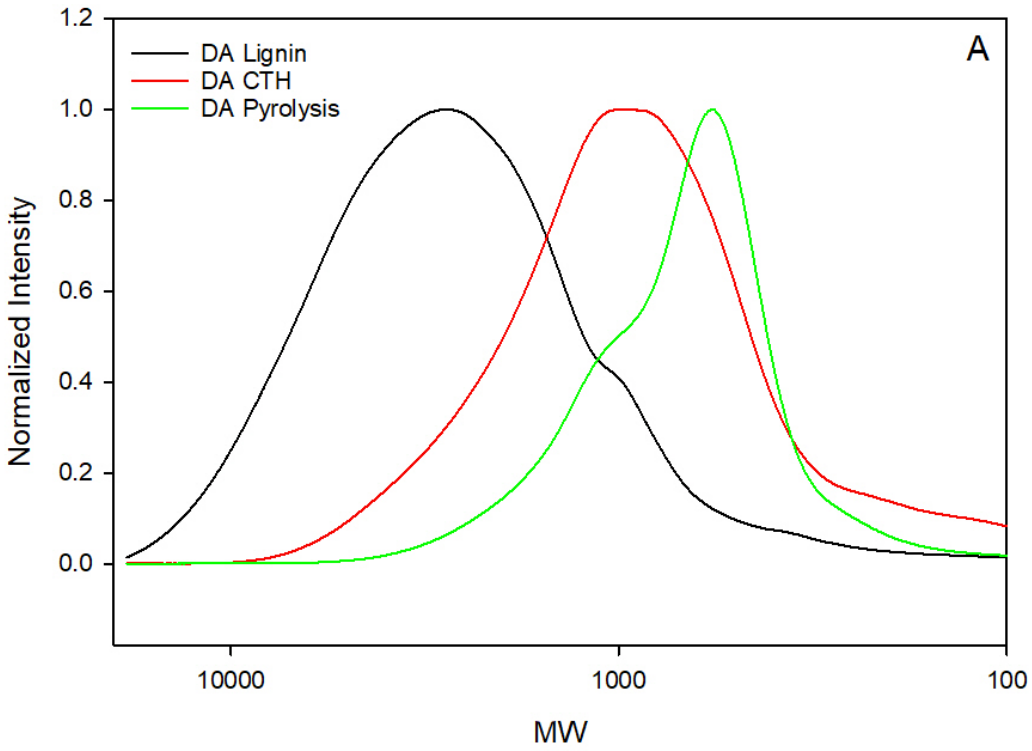
*2.2.5 Molecular Weight Distribution of Lignin Fractions*

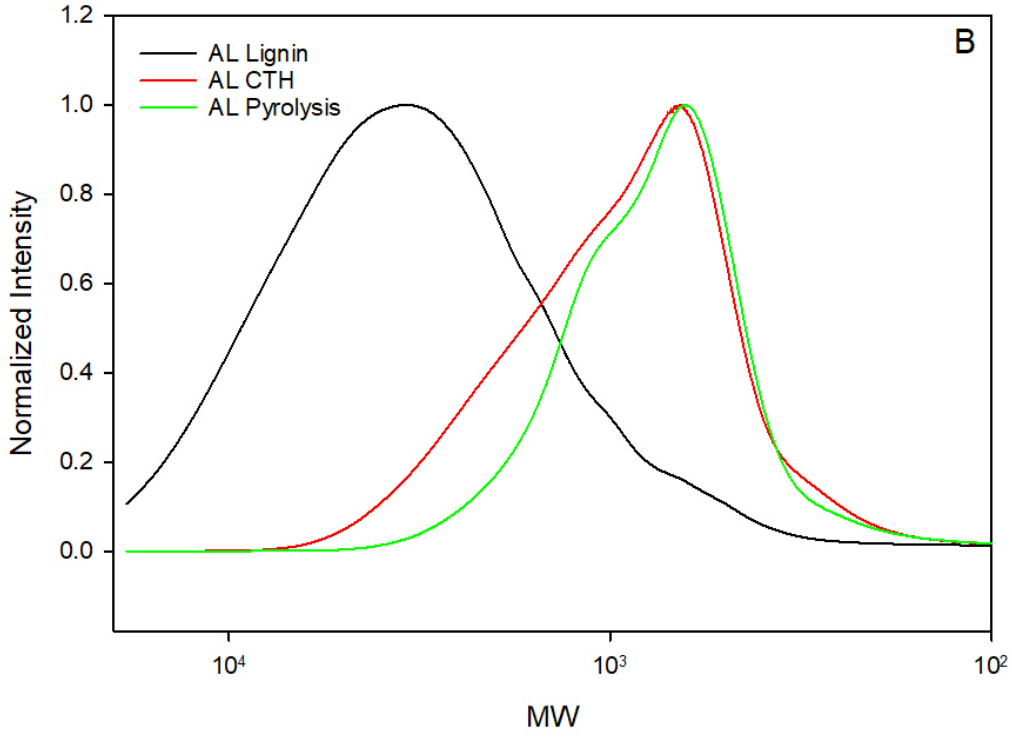
In order to better understand changes in the molecular weights distribution, the weight-average molecular weight ($M_w$) and number-average molecular weight ($M_n$) of the untreated lignin and the oil recovered from the sequential extraction are shown in

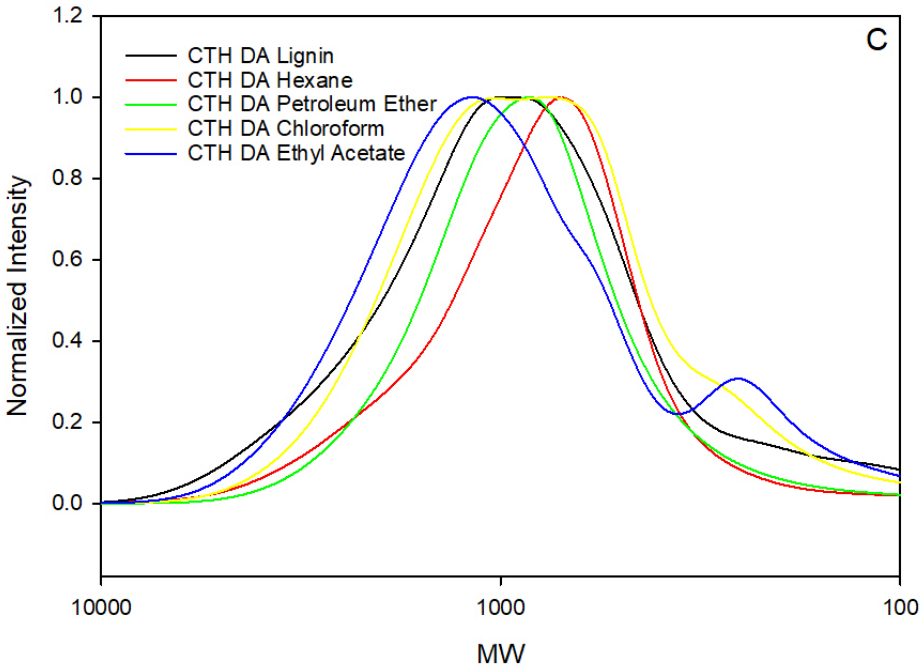
Table 8. In addition, the molecular weight distribution (MWD) profiles are illustrated in Figure 8. Comparing the MWD profiles of the unreacted lignin with CTH and pyrolysis lignin, the MWD curves shifted to later retention times (correspond to lower $M_w$ as shown in Figure 8 A & B), confirms lignin depolymerization via CTH and Pyrolysis. The $M_w$ of the DA lignin is 3,483 g/mol, while the CTH DA is 1,181 g/mol and the pyrolysis DA is 822 g/mol, indicating greater extent of lignin depolymerization caused by pyrolysis. The $M_w$ of the AL lignin is 4,347 g/mol, while the CTH and pyrolysis AL were 1,157 g/mol and 874 g/mol, respectively, illustrating the affinity of lignin depolymerization towards pyrolysis.

The $M_w$ slightly increase through the sequential extraction process for the DA. There are a few mechanics that could explain this. One is that the earlier solvents could be extracting compounds with lower $M_w$. It is also speculated that this $M_w$ increase through the process for DA could be lignin molecules are starting to re-polymerize. Pyrolysis reduced the average molecular weight of the lignin more than CTH. This finding is backed by a recent study showing that pyrolysis degraded the lignin fivefold compared to the starting corn stover sample (McClelland, Motagamwala et al. 2017). Untreated AL lignin had a PDI value of 2.56; after being subjected to CTH, or pyrolysis, the PDI value for the lignin oil dropped to 1.94 and 1.70 respectively. A lower PDI indicates greater uniformity for molecular weight within the mixture. The increase in PDI for DA demonstrated a wider span of $M_w$ after CTH and pyrolysis reaction, suggesting

that lignin depolymerization and re-condensation may occur in the same process.





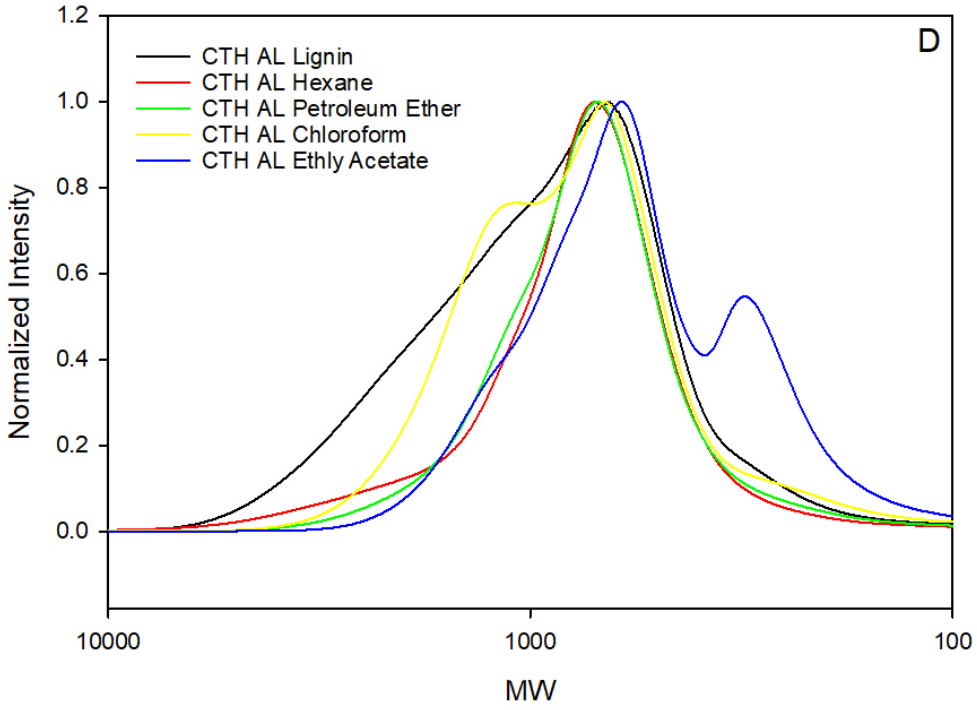




**Figure 8:** GPC spectra of raw and thermochemically processed **A)** DA and **B)** AL lignin and **C)** DA lignin's sequentially extracted fractions and **D)** AL lignin's sequentially extracted fractions resulted from CTH

**Table 8**: The molecular weight distribution of raw lignin and the lignin oils derived from CTH and pyrolysis and their sequentially extracted fractions

| | | Mn (g/mol) | Mw (g/mol) | PDI |
|---|---|---|---|---|
| **Raw lignin** | **DA** | 1525.4 | 3483.2 | 2.28 |
| | **AL** | 1701.1 | 4347.1 | 2.56 |
| **CTH DA** | **Bio Oil** | 389.4 | 1181.6 | 3.03 |
| | **Hexane** | 501.9 | 1018.1 | 2.03 |
| | **Petroleum Ether** | 620.7 | 1035.2 | 1.67 |
| | **Chloroform** | 454.2 | 1047.6 | 2.31 |
| | **Ethyl Acetate** | 452.6 | 1218.4 | 2.69 |
| | **Water + IPA Mix** | ND | ND | ND |
| **CTH AL** | **Bio Oil** | 597.0 | 1157.1 | 1.94 |
| | **Hexane** | 579.2 | 946.1 | 1.63 |
| | **Petroleum Ether** | 540.4 | 831.6 | 1.54 |
| | **Chloroform** | 527.4 | 933.0 | 1.77 |
| | **Ethyl Acetate** | 379.5 | 657.1 | 1.73 |
| | **Water + IPA Mix** | ND | ND | ND |
| **Pyro DA** | **Bio Oil** | 468.9 | 822.7 | 1.75 |
| | **Hexane** | 576.6 | 821.1 | 1.42 |
| | **Petroleum Ether** | 525.2 | 850.8 | 1.62 |
| | **Chloroform** | 519.8 | 896.4 | 1.72 |
| | **Ethyl Acetate** | 390.8 | 925.5 | 2.37 |
| | **Water + IPA Mix** | ND | ND | ND |
| **Pyro AL** | **Bio Oil** | 513.9 | 874.0 | 1.70 |
| | **Hexane** | 608.9 | 854.5 | 1.40 |
| | **Petroleum Ether** | 578.8 | 844.8 | 1.46 |
| | **Chloroform** | 553.5 | 904.5 | 1.63 |
| | **Ethyl Acetate** | 466.9 | 836.6 | 1.79 |
| | **Water + IPA Mix** | ND | ND | ND |

ND= not determined

## 2.4. Conclusions

From the composition analysis, glucan and xylan were observed in the DA and AL lignin samples and the lignin content was found to be lower than that of Kraft lignin.

FTIR results confirm that both DA and AL lignin samples have a more complex composition than Kraft lignin. This low lignin content in the samples is probably contributing to the decreased oil yields after pyrolysis and CTH. Differences in compound concentrations from GC/MS analysis suggest that lignin depolymerization products are affected by the pretreatment method and the breakdown process performed. For both lignin streams, pyrolysis produced a larger percentage of lignin oil yet, the only statistically significant difference was between the CTH and pyrolysis of DA lignin. CTH lignin oil was less polar compared to pyrolysis lignin oil, probably due to saturation of the derived compounds as a result of hydrogen. Given the polarity preferences, there is promise to design a better sequential extraction process extracting different compounds using solvents with an increasing polarity. Pyrolysis and CTH were both shown effectively reducing the average molecular weight of the lignin samples, but pyrolysis is more effective.

# CHAPTER 3: SCREENING ON LIGNIN MODEL COMPOUNDS AND EXTRACTED LIGNIN FRACTIONS FOR ANTIMICROBIAL PROPERTIES

## Introduction

The antimicrobial potentials of six model lignin compounds as well as the dilute acid and alkaline pretreatments lignin oils and their sequential extractives, were tested using a bioassay. The samples were assayed with three different microbes: *S. cerevisiae*, *E. coli*, and *L. amylovorus*. A plate reader was used to track the growth of the microbes by monitoring the OD 600 in the wells over the course of 36 hours. The OD 600 at maximal growth was compiled into heat-maps to compare antimicrobial effectiveness of different concentrations of model lignin compounds and the DA and AL lignin oil and the extractives.

## 3.1 Experimental

### *3.1.1 Materials*

The samples derived from enzymatic hydrolysis as well as the dilute acid and alkaline pretreatments on corn stover were provided by National Renewable Energy Laboratory (NREL). Guaiacol, vanillin, syringaldehyde, and 2,6-dimethoxyphenol were purchased from Sigma Aldrich. Vanillic acid and syringic acid were purchased from TCI America. The pyrolysis reaction oil came from Louisiana State University (LSU). NRRL, in Golden Colorado, Culture Collection provided the *Escherichia coli* (E. coli) (NRRL B-409), *Lactobacillus Amylovorus* (L. amylovorus) (B-4540), and the *Saccharomyces cerevisiae* (S. cerevisiae) (NRRL Y-567). The *L. amylovorus* broth was purchased from

BD. Fishersci supplied typtone, *S. cerevisiae* extract, and the D-Glucose. $KH_2PO_4$ was purchased from Sigma Aldrich and the peptone came from Alfa Aesar.

Monensin is an ionophore antibiotic mainly used in cattle feed. This study used Monensin as a control to see how it compares to lignin derived compounds.

### *3.1.2 Preparation of Inoculum*

All three microbes were propagated in liquid cultures for inoculating the well-plate culture. *L. amylovorus* was grown with a *L. amylovorus* MRS Broth at 37°C for 12 hours while stirred at 180 rpm. *S. cerevisiae* was grown using YPD medium and was incubated at 32°C for 12 hours at 180 rpm shaking speed. *E.coli* was grown in TGY medium for 12 hours and incubated at 37 °C and 180 rpm. The cultures were harvested using centrifugation at 4000 rpm for 10 minutes and re-suspended in a small amount of growth media and used as inoculum.

### *3.1.3 Cultivation in 48-well Plate*

Four concentrations on each mono-lignol compound were used to test for inhibitoy effects on the three different microbes: *S. cerevisiae*, *E. coli*, and *L. amylovorus*. These four concentrations were: 0 mg/mL, 0.1 mg/mL, 0.4 mg/mL, and 1.5 mg/mL. These concentrations were chosen to give a broad range that would aid in antimicrobial threshold predictions. These concentrations were based on of previous findings. A study done in 1979 tested eleven different compounds against a variety of microbes, including *S. cerevisiae* and *E. coli*. Depending on the compound, the range for antimicrobial effects were between 0.09 to 3 mg/mL for the *S. cerevisiae* and *E. coli* (Zemek, Košíková et al. 1979). All compounds at the four concentrations were dissolvable in ethanol and DMSO. Because the end application for these compounds is to be used in ethanol fermentation,

we chose to use ethanol. Each plate was cultured for 36 hours and at a set temperature of 32°C. The plates were stirred every 15 minutes for 30 seconds. The plate reader (Spectra Max M2 from Molecular Devices) took an optical density reading every 30 minutes at a wavelength of 600nm (Greenwood 1997).

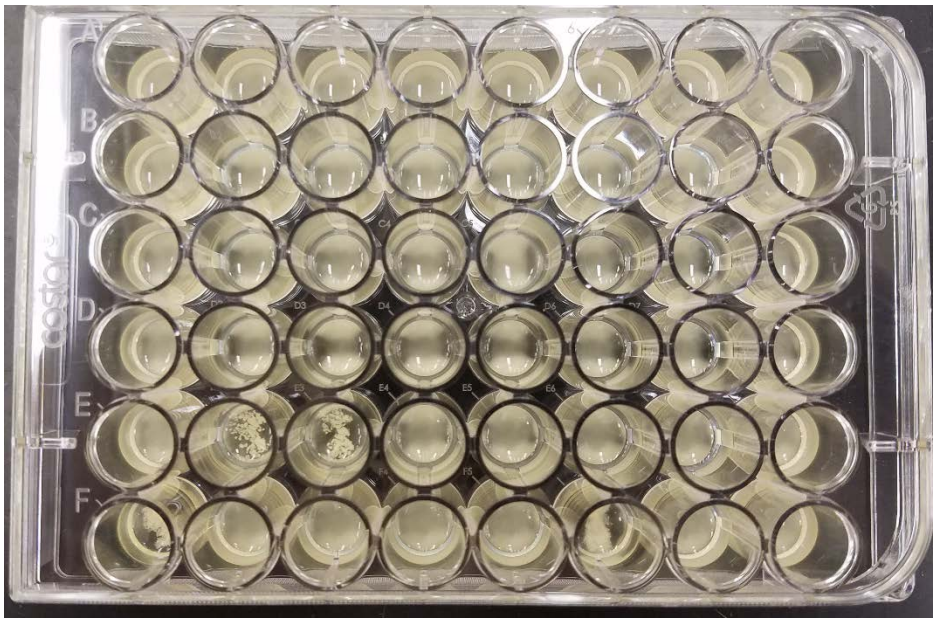



**Figure 9:** 48-well plate containing *S. cerevisiae* culture after 36 hours

*3.1.4 Heat-map and Antimicrobial Effect*

In order to create a heat-map, the 36-hour data from the plate reader were used. The change in OD was calculated for each well in duplicate. The control had the solvent ethanol and organism present. All three concentrations: 0.1 mg/mL, 0.4 mg/mL, and 1.5 mg/mL, were compared to the control, 0 mg/ml. This gave a percentage difference that was used to classify the strength of the antimicrobial effect. The heat-maps report the change in OD in in comparison to the control within each group of tested compounds. The dark green band, 1.00, represents no difference from control or, no antimicrobial effect. Moving down to the lighter green band, 0.80 - 0.99, which represents 80% - 99%

growth as compared to the control, or almost no antimicrobial effect. The bright yellow band, 0.60 – 0.79, represents a 60% - 79% growth as compared to the control or, a slight antimicrobial effect. The dark yellow band, 0.40 – 0.59, represents 40% - 59% growth as compared to the control or, stronger antimicrobial effect. The orange band, 0.20 – 0.39, represents 20% - 39% growth as compared to the control or, an antimicrobial effect. Lastly, the red band, 0.00 – 0.19, represents 0% to 19% growth as compared to the control, or a severe antimicrobial effect.

### *3.1.5 Statistical Analysis*

All experiments were conducted in duplicates or triplicates and the data are presented with means and standard deviations. The statistical analysis was performed by SAS® 9.4 (SAS Institute, Cary, NC, US), with a significance level of $P<0.05$ for all the data obtained from experiments.

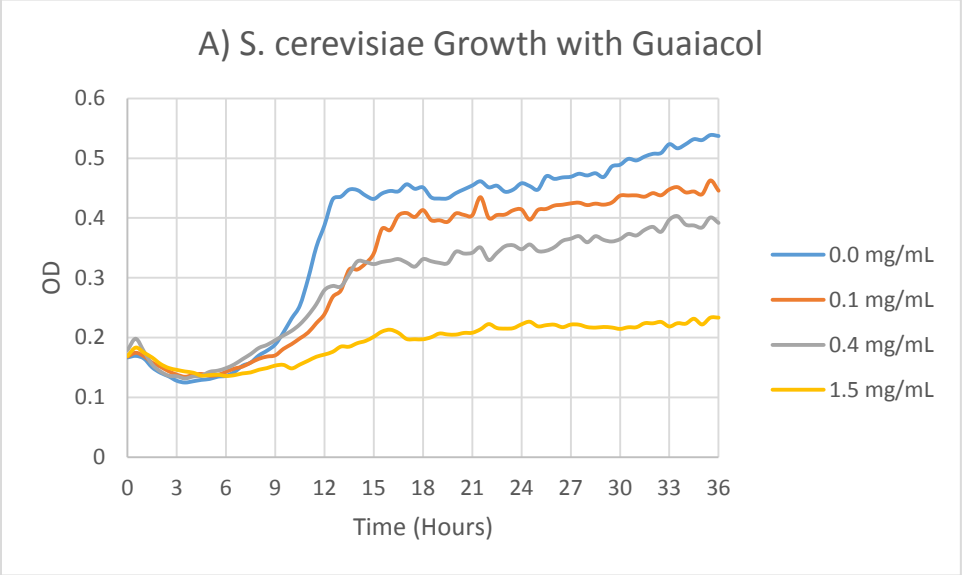
## 3.2 Results and Discussion

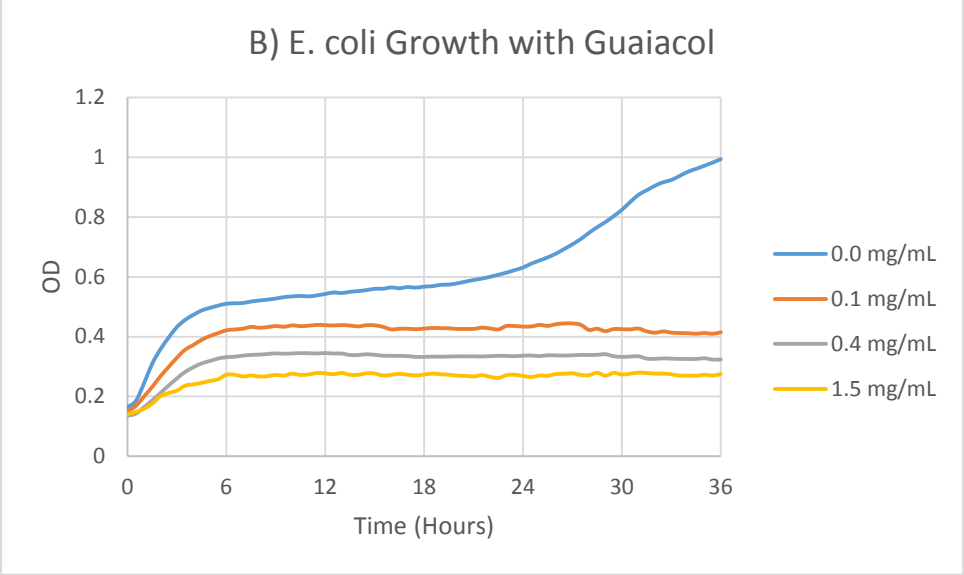
### *3.2.1 Growth Curves for Pure Lignin Model Compounds*

Figures 10 A-C show typical growth curves for the three microbes with the addition of a lignin mono compound guaiacol. As the concentration of guaiacol increased, its inhibitory factor also increased. With a concentration of 1.5 mg/ml of guaiacol the *S. cerevisiae* had almost no growth at all (0 – 19% growth). Whereas the inhibitor factor of 0.4 mg/mL was between 40-59%.

*L. amylovorus* is to be more resistant than *S. cerevisiae* to guaiacol. The first three concentrations (0.0-0.4 mg/mL) of guaiacol had no noticeable effect on the growth of *L. amylovorus*. It is not until a concentration of 1.5 mg/ml was reached that any inhibitory

effect was observed. A heat-map displaying all the pure model lignin compounds tested against: *S. cerevisiae*, *E. coli*, and *L. amylovorus* is displayed in Figure 9.



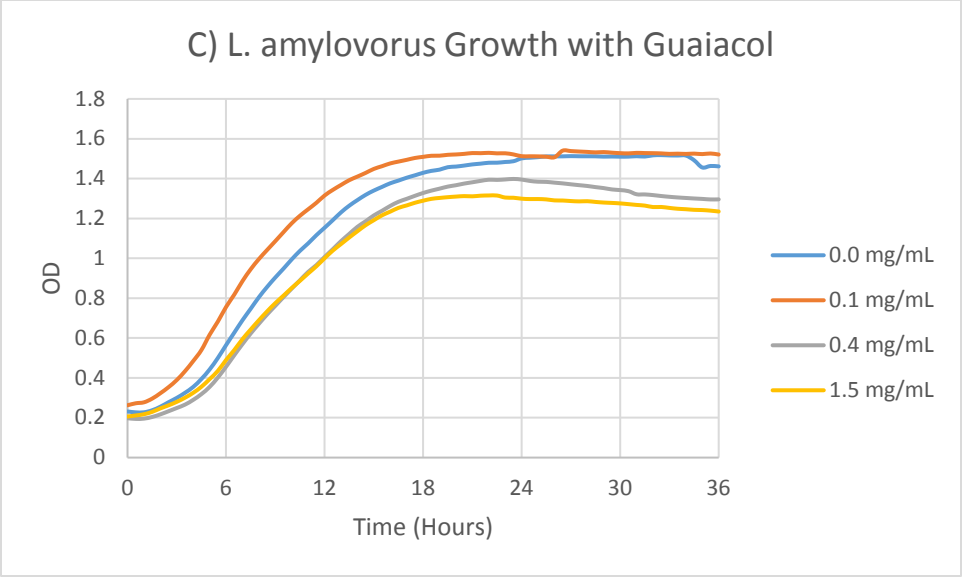



**Figure 10:** Growth curves for **A)** *S. cerevisiae*, **B)** E. coli, and **C)** L. amylovorus growth incubated at different concentrations of guaiacol

*3.2.2 Heat-maps for Pure Model Lignin Compounds*

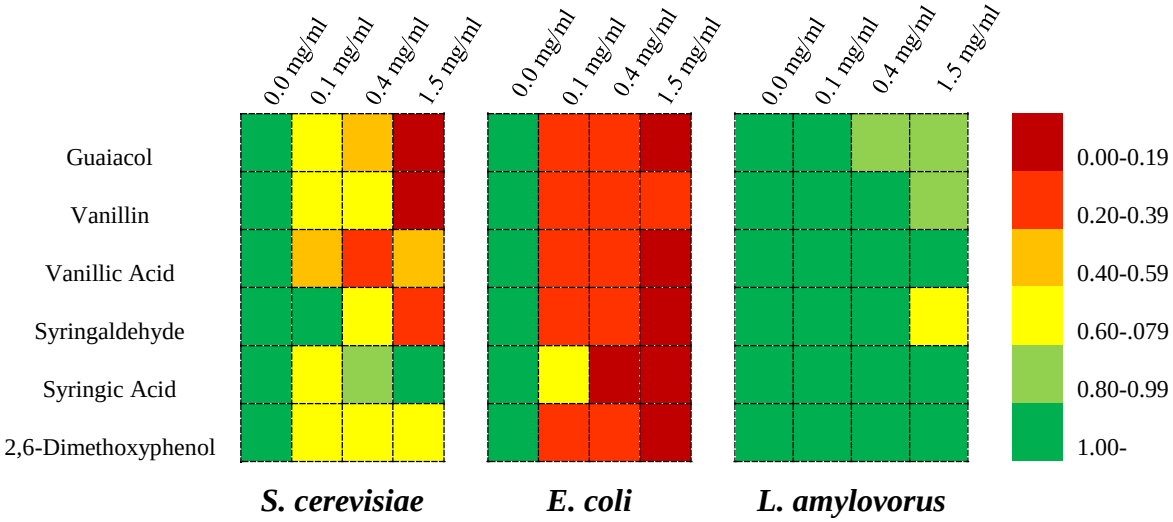



**Figure 11:** Heat-maps showing the inhibition of 6 lignin model compounds on the growth of *S. cerevisiae*, E. coli, and L. amylovorus

The heat-maps in Figure 11 were constructed to better show how each of the model compounds inhibits the growth of microbes. Looking at these heat-maps, it was not expected that *L. amylovorus* would be so robust, nor was it expected that *E.coli* would be so susceptible to the lignin derived compounds.

Some compounds had an inhibitory effect on *E.coli* and did not exhibit that same effect on *S. cerevisiae*: syringaldehyde or syringic acid. 1.5 g/L of syringaldehyde showed some inhibitory effect on *L. amylovorus*. At that loading syringaldehyde also inhibited the growth of *S. cerevisiae*. Model lignin compounds syringaldehyde and syringic acid have the best selective inhibit against *E. coli* compared to *S. cerevisiae*. None of the model lignin compounds investigated had inhibitory effects on *L. amylovorus* when compared to *S. cerevisiae*. The data for the heat-maps and the additional growth curves for model lignin compounds can be found in Appendix E and G.

Monensin has been shown to have selectivity inhibition against *L. amylovorus* but does not inhibit the growth of *S. cerevisiae* (Oliva Neto, Lima et al. 2014). An experiment to confirm this claim was conducted and data are shown in Appendices D. Our test revealed that Monensin at the lowest concentrations exhibited the maximum amount of growth inhibition against *L. amylovorus.* Increasing concentration did inhibit yeast growth until the maximum concentration was reached. It is unsure as to why yeast had no inhibition at the maximum concentration of Monensin.

### *3.2.3 Growth Curves for Sequentially Extracted Lignin Fractions*

Figures 12 A and B show the growth curves for *E. coli* with the addition of CTH DA and AL lignin oils. For DA lignin oil, an inhibitory effect began to take effect at the lowest concentration of 0.1 mg/ml. The other two concentrations, 0.4 mg/ml and 1.5 mg/ml, do not show much difference from the lowest concentration. For AL lignin oil, no inhibitor factor is present for any of the concentrations. When comparing the CTH DA and AL oils, only DA pretreated lignin shows any potential to have antimicrobial

properties. The data for the heat-maps and the additional growth curves for extracted fractionations can be found in Appendix C and D

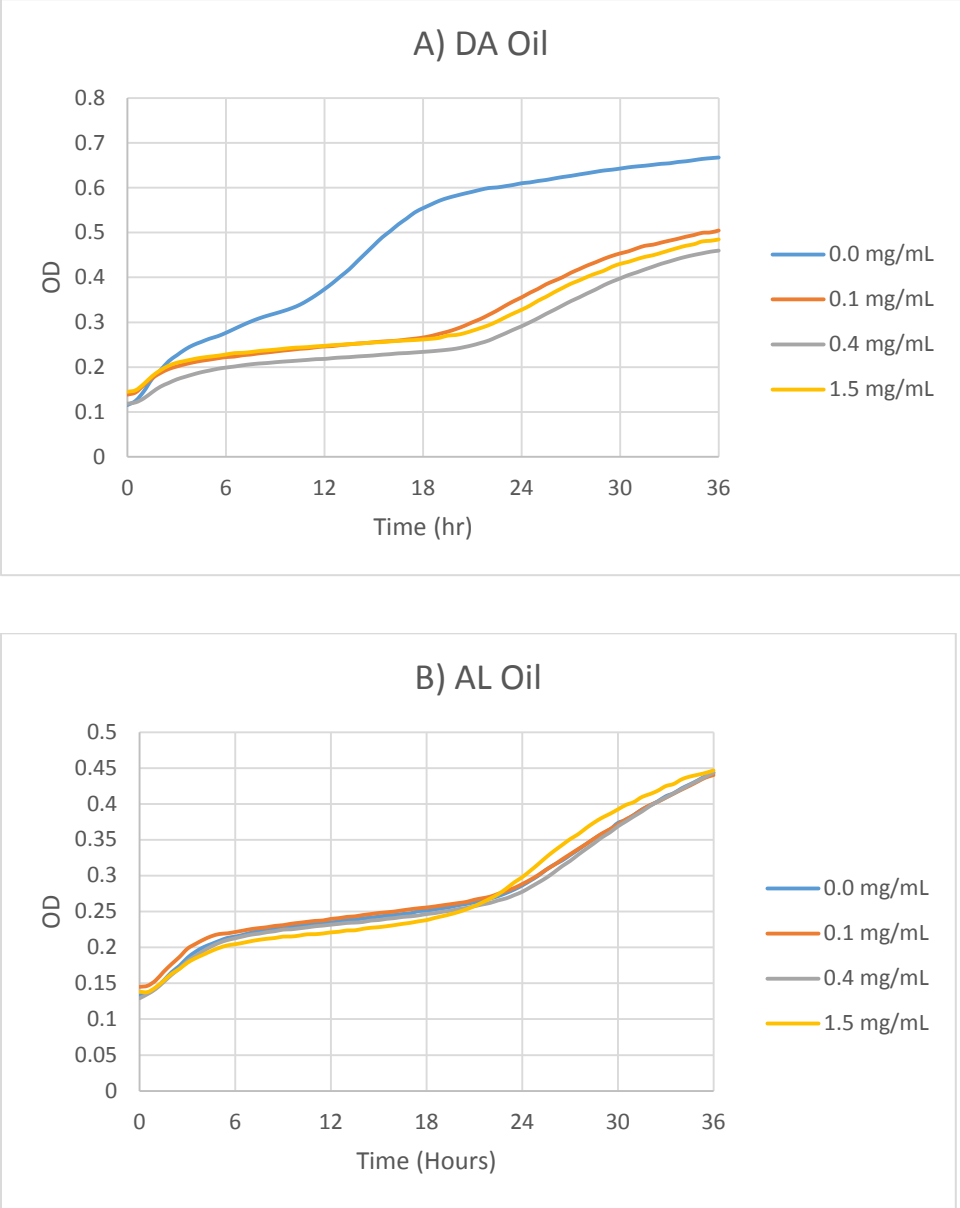



**Figure 12:** Growth curves for E. coli incubated at different concentrations of CTH **A)** DA and **B)** AL lignin oil

*3.2.4 Heat-maps from Sequentially Extracted Lignin Fractions*

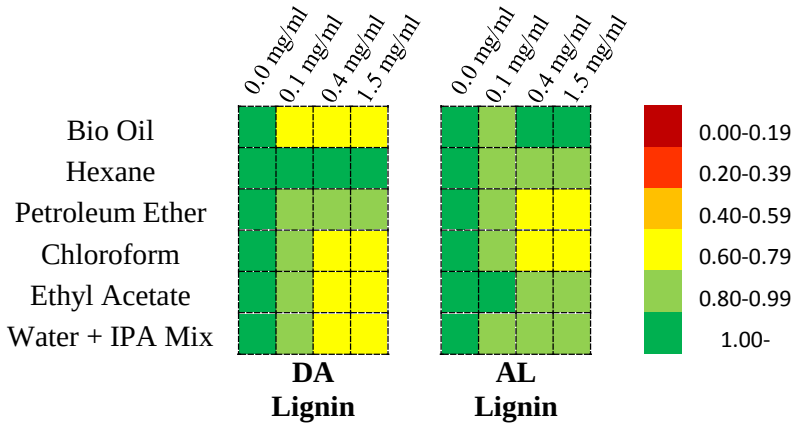



**Figure 13:** Heat-maps showing the inhibition of E.coli against CTH fractionations

The heat-maps in Figure 13 were assembled to better represent how each of the fractions from the sequential extraction process inhibits the growth of a microbe. It was not expected that DA lignin and AL lignin would be different, although these two lignin streams have the chloroform fraction exhibiting the same antimicrobial effect. Chloroform for both DA and AL lignin stunts the growth of *E. coli* to between 0.60 – 0.79 of its control growth at 0.4 gm/ml and 1.5 mg/ml. None of the fractionations for either lignin stunted the growth of *E. coli* below 0.60. Looking at the DA lignin bio oil, it can be observed that all three concentrations performed just as well as the sequential extraction concentrations, so the sequential extraction is not necessary if antimicrobial properties are desired. From this pilot data, CTH fractionations for both DA and AL lignin do not exhibit a noticeable antimicrobial properties against *E. coli*. Further research needs to be conducted to determine if DA and AL lignin have any antimicrobial effect against *S. cerevisiae* and *L. amylovorus*.

### *3.3 Conclusions*

The model lignin compounds had no effect on the growth of *L. amylovorus.* Yet, all of the model lignin compounds showed effects on the growth of *E. coli.* This is most likely attributed to the differences in cell wall structure and cell wall composition between the two microbes. The inhibitory effect on *S. cerevisiae* was dependent on the type on model lignin compound. With the S lignin derived model compounds, syringakdehyde, syringic acid, and 2,6-dimethoxyphenol, visually showing less inhibitory effects compared to model compounds derived from G lignin. S lignin model compounds show a selective effect for *S. cerevisiae* compared to *E. coli.* Inhibiting the growth of *E. coli* more than the growth of *S. cerevisiae.* Syringic acid shows the best selective antimicrobial property when comparing *S. cerevisiae* to *E. coli*.

When comparing the CTH DA and CTH AL oils, DA lignin oils performs better for antimicrobial properties against *E. coli*. CTH DA has the oil as well as three fractionation phases, chloroform, ethyl acetate, and water + IPA mix that all show an inhibitory factor of 60-79% at the 0.4 and 1.5 mg/mL. On the other hand, CTH AL only shows two fractionation phases, petroleum ether and chloroform, that show an inhibitory factor of 60-79% at the concentrations of 0.4 and 1.5 mg/ mL. Further research needs to be conducted to fully understand the effect of fractionation on the antimicrobial properties of lignin.

# 4. FURTHER RESEARCH

## *4.1 Pyrolysis and Catalytic Transfer Hydrogenolysis*

There are a few areas of further research within these two breakdown areas that should be examined. A full mass balance should be conducted. The attempted mass balance in this study is not sufficient. Tracking how much lignin went into one of these processes and where it ended up could help with research in establishing better breakdown methods. How the percentage of glucose in the lignin sample affects the efficiency of the process should be experimented to see if glucose is a factor that leads to more or less oil yields.

More experiments of both pyrolysis and CTH need to be conducted to establish if there is a statistical difference between the different lignin streams and/or the breakdown processed themselves. Further calculations and/or research should be conducted to establish solubility of the lignin fractionations.

Scaled-up versions of these thermochemical process should undergo more testing. This would help to establish if these processes are even feasible for commercial use. Larger qualities of lignin should be run to see how the heat transfer of these process is effected when the quality of lignin used is larger than eight grams. Conducting an energy analysis on these two processes would provide valuable information. This would help to determine how much work it takes to break down a set amount of lignin and to see if this relationship is linear or exponential when scaling up.

## *4.2 Antimicrobial Testing*

These results from this testing are interesting and should spark further research. Further experiments need to be conducted to establish if an antimicrobial relationship

between the lignin streams after sequential extraction and the microbes of *S. cerevisiae* and *L. amylovorus* exists. All microbial tests in this study should be repeated with smaller intervals between the concentrations. This would help to establish exactly which concentration is the most effective and establish a threshold concentration that each microbe would still grow normally. Other microbes should also be explored to see if there are other applications for the fractionations of lignin.

Batch testing of different microbes should be explored to see how the selective antimicrobial properties of lignin play out in the presences of more than one microbe.

# APPENDICES

*Appendices A. Sequential Extraction Solvent Percentages*

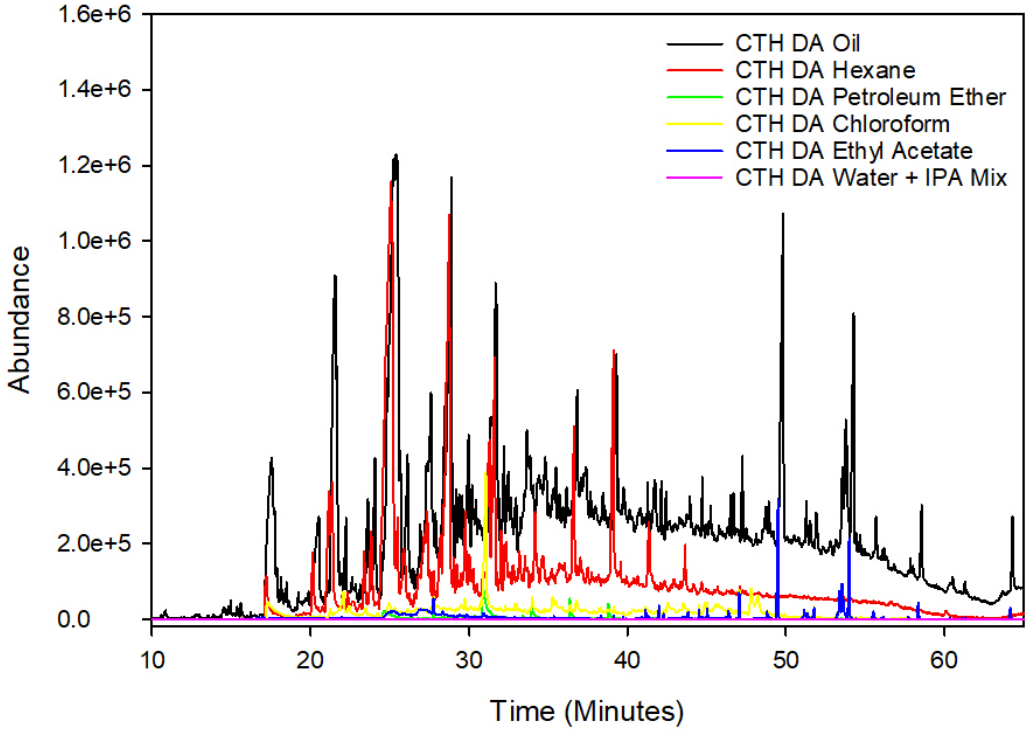
| CTH-DA | | | | | | |
|---|---|---|---|---|---|---|
| **Compound Name** | **Bio Oil'** | **Hexane'** | **Petroleum Ether'** | **Chloroform'** | **Ethyl Acetate'** | **Water+ IPA Mix** |
| Phenol | 4.52% | | | | | |
| Phenol, 3-methyl- | 4.31% | | | | | |
| Phenol, 2-methoxy- | 3.51% | 2.34% | | | | |
| Phenol, 4-ethyl- | 19.39% | 36.47% | | | | |
| Phenol, 3-ethyl-5-methyl- | 2.15% | | | | | |
| Phenol, 4-ethyl-2-met | 7.12% | 17.94% | | | | |
| Phenol, 2-methoxy-4-propyl- | 2.05% | 14.70% | | | | |
| Phenethylamine, 2,4,5-trimethoxy-a-methyl- | 2.35% | | | | | |
| Phenol, 4-(3-hydroxy-1-propenyl)-2-methoxy- | 2.03% | | | | | |
| p-Cresol | | 2.79% | | | | |
| Phenol, 2,3-dimethyl- | | 2.20% | | | | |
| Creosol | | 2.83% | | | | |
| Phenol, 2,6-dimethoxy | | 12.84% | | | | |
| Benzene, 1,2,3-trimet | | 3.54% | | | | |
| **Total** | **47.42%** | **95.64%** | 0% | 0% | 0% | 0% |

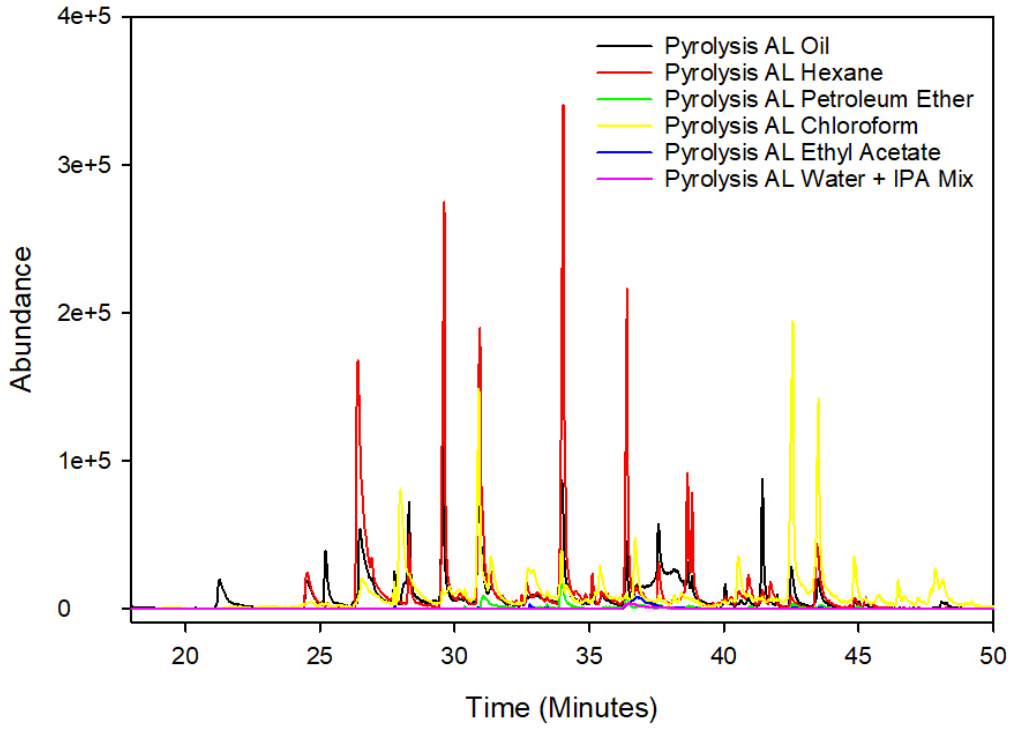
| Pyrolysis-DA | | | | | | |
|---|---|---|---|---|---|---|
| **Compound Name** | **Bio Oil'** | **Hexane'** | **Petroleum Ether'** | **Chloroform'** | **Ethyl Acetate'** | **Water + IPA Mix** |
| Furfural | 6.52% | | | | | |
| Phenol | 8.19% | | | | | |
| Phenol, 2-methoxy- | 18.33% | | | | | |
| Phenol, 4-ethyl- | 10.03% | | | | | |
| Benzaldehyde, 2-methy | 18.04% | | | | | |
| Phenol, 4-ethyl-2-met | 5.70% | | | | | |
| 2-Methoxy-4-vinylphenol | 16.46% | 30.10% | | | | |
| Phenol, 2,6-dimethoxy- | 16.74% | 34.43% | | | | |

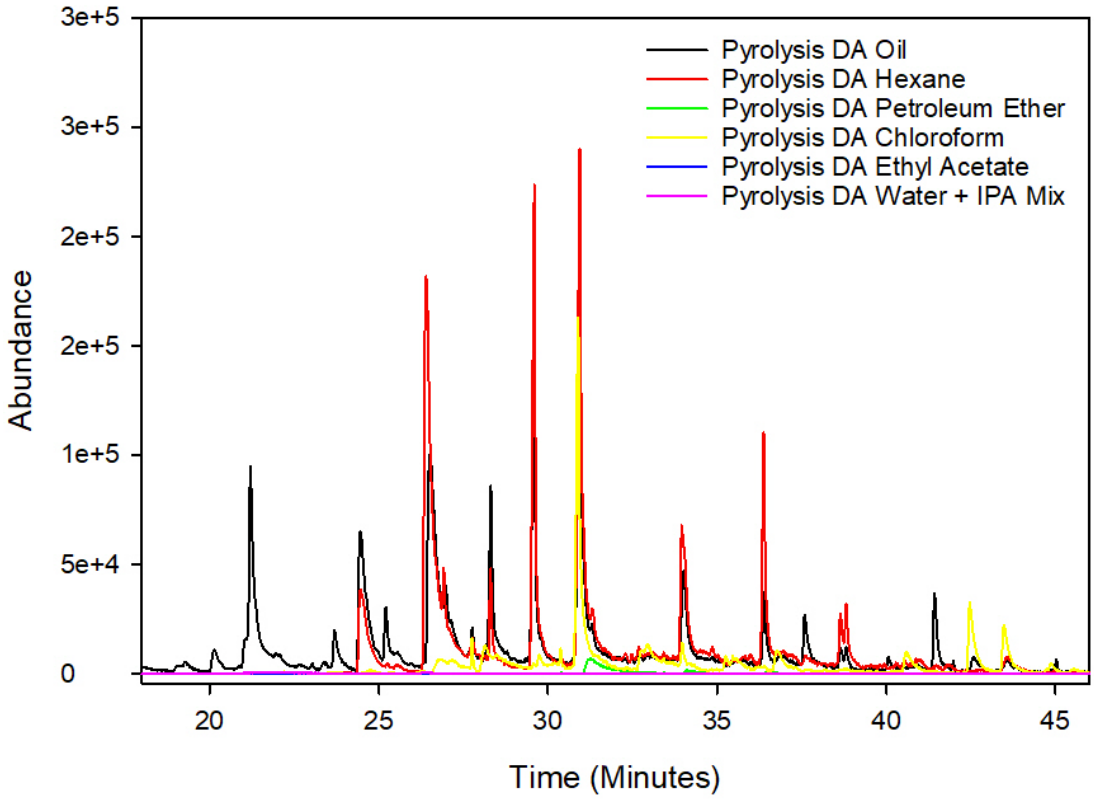
| | | | | | | |
|---|---|---|---|---|---|---|
| Benzofuran, 2,3-dihydro | | 35.47% | | | | |
| ***Total*** | ***100%*** | ***100%*** | 0% | 0% | 0% | 0% |

| Pyrolysis-AL | | | | | | |
|---|---|---|---|---|---|---|
| **Compound Name** | **Bio Oil'** | **Hexane'** | **Petroleum Ether'** | **Chloroform'** | **Ethyl Acetate'** | **Water + IPA Mix** |
| Phenol, 2-methoxy- | 15.60% | | | | | |
| Phenol, 4-ethyl-2-methoxy- | 14.12% | | | | | |
| 2-Methoxy-4-vinylphenol | 22.06% | 23.82% | | | | |
| Phenol, 2,6-dimethoxy- | 16.72% | 17.88% | | | | |
| Phenol, 4-methoxy-3-(methoxymethyl)- | 14.82% | 18.91% | | | | |
| Phenol, 2,6-dimethoxy-4-(2-propenyl)- | 10.60% | | | | | |
| Phenol, 2-methoxy-4-(1-propenyl)- | | 13.34% | | | | |
| Benzene, 1,2,3-trimethoxy-5-methyl- | | 11.06% | | | | |
| Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)- | | | 100% | | | |
| ***Total*** | ***93.91%*** | ***85.01%*** | ***100%*** | 0% | 0% | 0% |

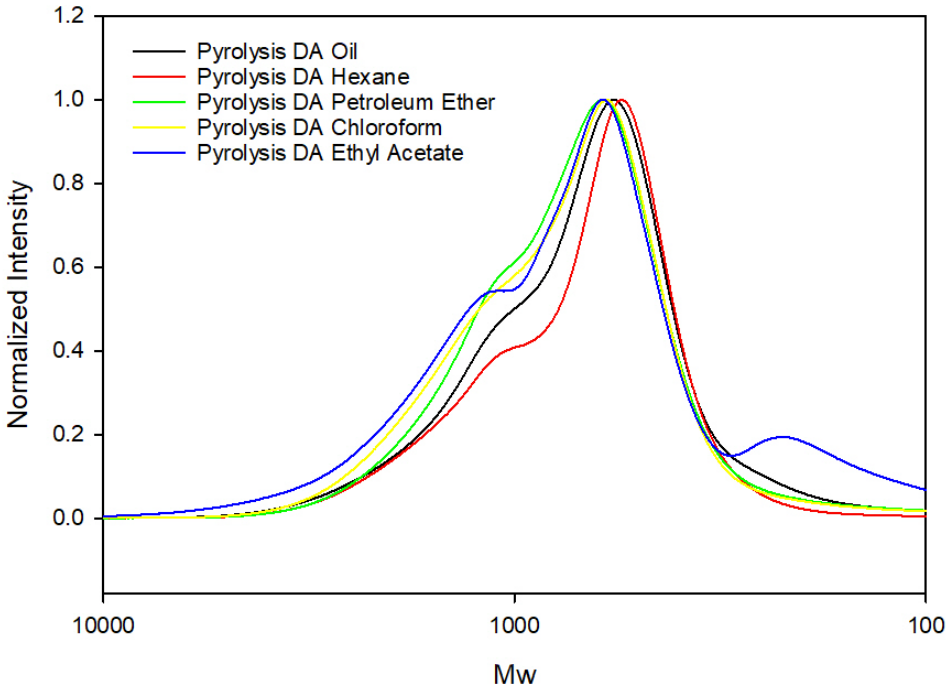
*Appendices B. GC/MS Chromatography of Extracted Fractionations*

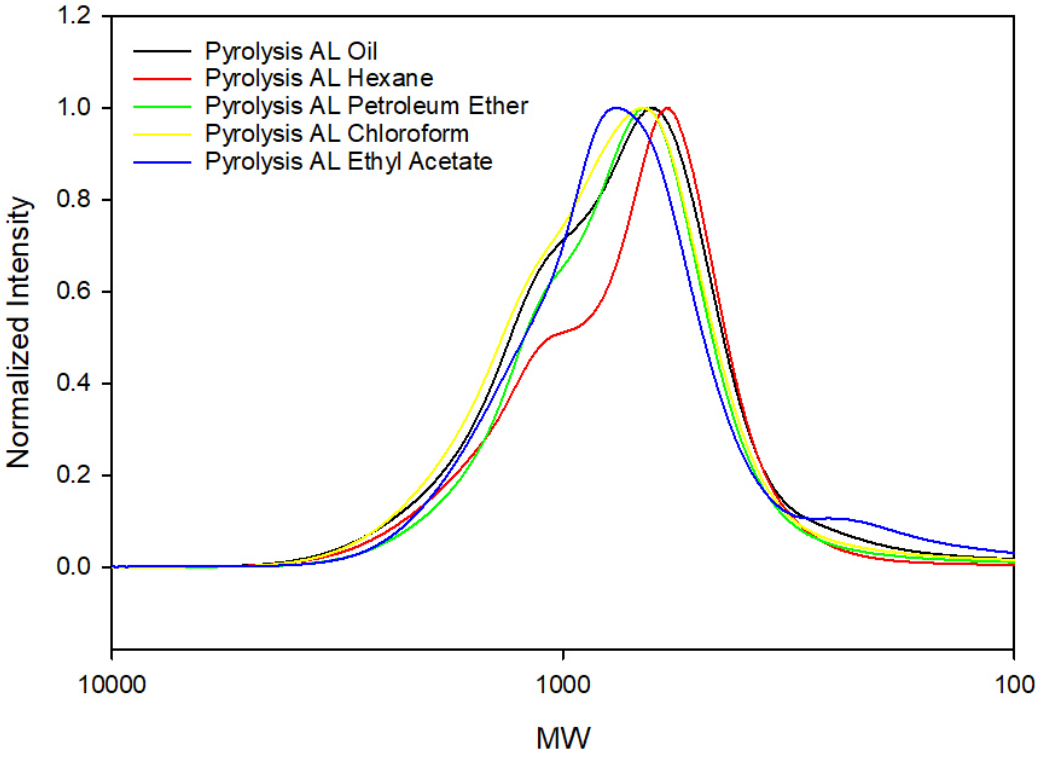



Pyrolysis DA Oil
Pyrolysis DA Hexane
Pyrolysis DA Petroleum Ether
Pyrolysis DA Chloroform
Pyrolysis DA Ethyl Acetate
Pyrolysis DA Water + IPA Mix
Abundance
3e+5
3e+5
2e+5
2e+5
1e+5
5e+4
0
20
25
30
35
40
45
Time (Minutes)

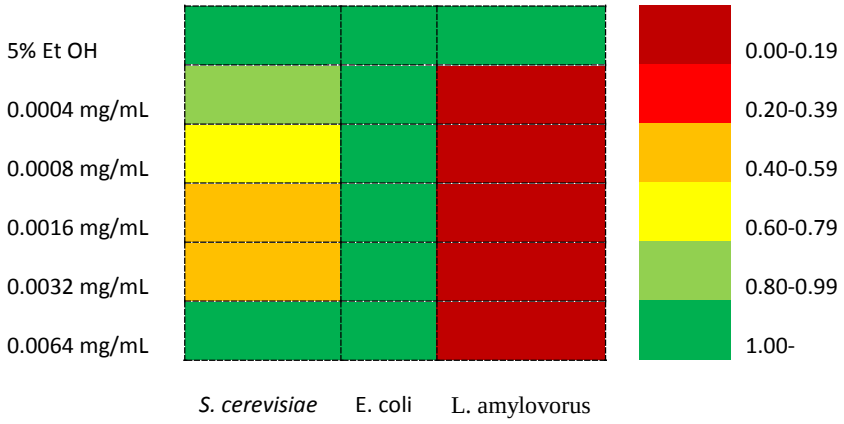
*Appendices C. GPC Spectra of Extracted Fractionations*



*Appendices D. Data and Heat-maps for Monensin*

| | 5% EtOH | 0.0004 | 0.0008 | 0.0016 | 0.0032 | 0.0064 |
|---|---|---|---|---|---|---|
| *S. cerevisiae* | 0.3731 | 0.4157 | 0.2728 | 0.2542 | 0.2301 | 0.5665 |
| *S. cerevisiae* | 0.3586 | 0.2421 | 0.1905 | 0.1165 | 0.1679 | 0.4746 |
| *S. cerevisiae* Avg. | 0.36585 | 0.3289 | 0.23165 | 0.18535 | 0.199 | 0.52055 |
| *S. cerevisiae* % | 1.000 | 0.899 | 0.633 | 0.507 | 0.544 | 1.423 |
| E.Coli | 0.2345 | 0.2714 | 0.2806 | 0.2708 | 0.277 | 0.3748 |
| E.Coli | 0.2437 | 0.2723 | 0.2765 | 0.2721 | 0.2858 | 0.291 |
| E.Coli Avg. | 0.2391 | 0.27185 | 0.27855 | 0.27145 | 0.2814 | 0.3329 |
| E.Coli % | 1.000 | 1.137 | 1.165 | 1.135 | 1.177 | 1.392 |
| L. amylovorus | 1.2493 | 0.0883 | 0.0911 | 0.2321 | 0.0855 | 0.1139 |
| L. amylovorus | 1.2644 | 0.0881 | 0.0527 | 0.0957 | 0.0643 | 0.1182 |
| L. amylovorus Avg. | 1.25685 | 0.0882 | 0.0719 | 0.1639 | 0.0749 | 0.11605 |
| Lacto % | 1.000 | 0.070 | 0.057 | 0.130 | 0.060 | 0.092 |



*Appendices E. Data from Microbes against Model Lignin Compounds*

*S. cerevisiae* incubation with model lignin compounds

| | 0 mg/ml | 0.1 mg/ml | 0.4 mg/ml | 1.5 mg/ml |
|---|---|---|---|---|
| Guaiacol | 0.4354 | 0.2868 | 0.2529 | 0.0998 |
| Guaiacol | 0.3054 | 0.2683 | 0.1726 | 0.0287 |
| Guaiacol Avg. | 0.3704 | 0.27755 | 0.21275 | 0.06425 |
| Guaiacol % | 1.000 | 0.749 | 0.574 | 0.173 |
| Vanillin | 0.2598 | 0.2118 | 0.1839 | 0 |
| Vanillin | 0.3642 | 0.2112 | 0.224 | 0.0179 |
| Vanillin Avg. | 0.312 | 0.2115 | 0.20395 | 0.00895 |
| Vanillin % | 1.000 | 0.678 | 0.654 | 0.029 |
| Vanillic acid | 0.309 | 0.2236 | 0.1165 | 0.3826 |
| Vanillic acid | 0.8484 | 0.2496 | 0.1418 | 0.1072 |
| Vanillic acid Avg. | 0.5787 | 0.2366 | 0.12915 | 0.2449 |
| Vanillic acid % | 1.000 | 0.409 | 0.223 | 0.423 |
| Syringaldehyde | 0.2988 | 0.3894 | 0.2885 | 0.1237 |
| Syringaldehyde | 0.6554 | 0.5757 | 0.444 | 0.0852 |
| Syringaldehyde Avg. | 0.4771 | 0.48255 | 0.36625 | 0.10445 |
| Syringaldehyde % | 1.000 | 1.011 | 0.768 | 0.219 |
| 2,6-dimethoxyphenol | 0.4165 | 0.2464 | 0.2751 | 0.1812 |
| 2,6-dimethoxyphenol | 0.9671 | 0.4505 | 0.4563 | 0.3858 |
| 2,6-dimethoxyphenol Avg. | 0.6918 | 0.34845 | 0.3657 | 0.2835 |
| 2,6-dimethoxyphenol % | 1.000 | 0.504 | 0.529 | 0.410 |
| Syringic acid | 0.3303 | 0.2391 | 0.3248 | 0.3925 |
| Syringic acid | 0.5564 | 0.4316 | 0.5302 | 0.5311 |
| Syringic acid Av.g | 0.44335 | 0.33535 | 0.4275 | 0.4618 |
| Syringic acid % | 1.000 | 0.756 | 0.964 | 1.042 |

*E. coli* incubation with model lignin compounds

| | 0 mg/ml | 0.1 mg/ml | 0.4 mg/ml | 1.5 mg/ml |
|---|---|---|---|---|
| Syringaldehyde | 0.9829 | 0.2471 | 0.1997 | 0.1303 |
| Syringaldehyde | 0.9557 | 0.2972 | 0.2352 | 0.15 |
| Syringaldehyde Avg. | 0.9693 | 0.27215 | 0.21745 | 0.14015 |
| Syringaldehyde % | 1.000 | 0.281 | 0.224 | 0.145 |
| Syringic acid | 0.9777 | 0.3209 | 0.19 | 0.1735 |
| Syringic acid | 0.9468 | 0.882 | 0.1821 | 0.1048 |
| Syringic acid Av.g | 0.96225 | 0.60145 | 0.18605 | 0.13915 |
| Syringic acid % | 1.000 | 0.625 | 0.193 | 0.145 |
| 2,6-dimethoxyphenol | 0.9739 | 0.3384 | 0.1837 | 0.069 |
| 2,6-dimethoxyphenol | 0.9683 | 0.2518 | 0.2079 | 0.1604 |

| | | | | |
|---|---|---|---|---|
| 2,6-dimethoxyphenol Avg. | 0.9711 | 0.2951 | 0.1958 | 0.1147 |
| 2,6-dimethoxyphenol % | 1.000 | 0.304 | 0.202 | 0.118 |
| Vanillic acid | 0.9841 | 0.4362 | 0.2518 | 0.0656 |
| Vanillic acid | 0.9612 | 0.3355 | 0.2088 | 0.0335 |
| Vanillic acid Avg. | 0.97265 | 0.38585 | 0.2303 | 0.04955 |
| Vanillic acid % | 1.000 | 0.397 | 0.237 | 0.051 |
| Vanillin | 0.9857 | 0.3187 | 0.2446 | 0.4193 |
| Vanillin | 0.9862 | 0.313 | 0.217 | 0.1219 |
| Vanillin Avg. | 0.98595 | 0.31585 | 0.2308 | 0.2706 |
| Vanillin % | 1.000 | 0.320 | 0.234 | 0.274 |
| Guaiacol | 0.7603 | 0.2287 | 0.2009 | 0.1281 |
| Guaiacol | 0.8992 | 0.3 | 0.176 | 0.1411 |
| Guaiacol Avg. | 0.82975 | 0.26435 | 0.18845 | 0.1346 |
| Guaiacol % | 1.000 | 0.319 | 0.227 | 0.162 |

*L. amylovorus* incubation with model lignin compounds

| | 0 mg/ml | 0.1 mg/ml | 0.4 mg/ml | 1.5 mg/ml |
|---|---|---|---|---|
| Syringaldehyde | 1.124 | 1.2116 | 1.1243 | 0.247 |
| Syringaldehyde | 1.0957 | 1.2557 | 1.2224 | 1.0907 |
| Syringaldehyde Avg. | 1.10985 | 1.23365 | 1.17335 | 0.66885 |
| Syringaldehyde % | 1 | 1.111547 | 1.057215 | 0.602649 |
| Syringic acid | 1.0557 | 1.331 | 1.3309 | 1.3769 |
| Syringic acid | 1.1495 | 1.2682 | 1.1985 | 1.2998 |
| Syringic acid Av.g | 1.1026 | 1.2996 | 1.2647 | 1.33835 |
| Syringic acid % | 1 | 1.178669 | 1.147016 | 1.213813 |
| 2,6-dimethoxyphenol | 1.0958 | 1.3561 | 1.2161 | 1.2175 |
| 2,6-dimethoxyphenol | 1.1474 | 1.1786 | 1.2426 | 1.2492 |
| 2,6-dimethoxyphenol Avg. | 1.1216 | 1.26735 | 1.22935 | 1.23335 |
| 2,6-dimethoxyphenol % | 1 | 1.129948 | 1.096068 | 1.099634 |
| Vanillic acid | 1.0585 | 1.3338 | 1.2803 | 1.339 |
| Vanillic acid | 1.1801 | 1.2899 | 1.3746 | 1.3093 |
| Vanillic acid Avg. | 1.1193 | 1.31185 | 1.32745 | 1.32415 |
| Vanillic acid % | 1 | 1.172027 | 1.185964 | 1.183016 |
| Vanillin | 1.1886 | 1.1963 | 1.2284 | 1.2248 |
| Vanillin | 1.2296 | 1.2715 | 1.2125 | 1.1578 |
| Vanillin Avg. | 1.2091 | 1.2339 | 1.22045 | 1.1913 |
| Vanillin % | 1 | 1.020511 | 1.009387 | 0.985278 |
| Guaiacol | 1.2772 | 1.4046 | 1.2196 | 1.2077 |
| Guaiacol | 1.1826 | 1.1121 | 0.977 | 0.8567 |
| Guaiacol Avg. | 1.2299 | 1.25835 | 1.0983 | 1.0322 |
| Guaiacol % | 1 | 1.023132 | 0.892999 | 0.839255 |

*Appendices F. Data from Microbes against Lignin Fractionations*

*E. coli* incubation with CTH DA fractionation

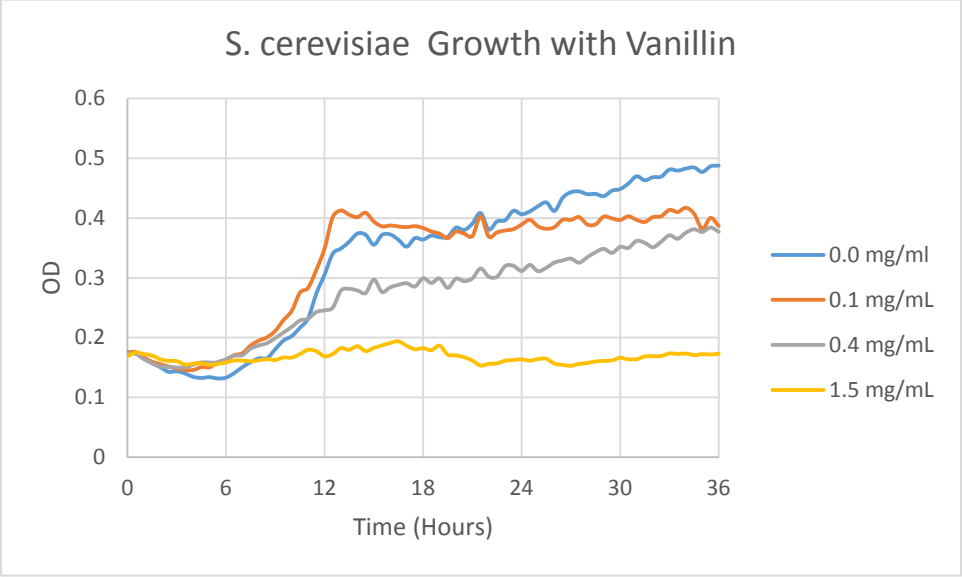
| | 0 mg/ml | 0.1 mg/ml | 0.4 mg/ml | 1.5 mg/ml |
|---|---|---|---|---|
| Hexane | 0.3223 | 0.3975 | 0.4316 | 0.4227 |
| Hexane | 0.4701 | 0.4123 | 0.3675 | 0.369 |
| Hexane Avg. | 0.3962 | 0.4049 | 0.39955 | 0.39585 |
| Hexane % | 1.000 | 1.022 | 1.008 | 0.999 |
| Petroleum Ether | 0.3987 | 0.3407 | 0.3419 | 0.3522 |
| Petroleum Ether | 0.3705 | 0.3636 | 0.3311 | 0.3502 |
| Petroleum Ether Avg. | 0.3846 | 0.35215 | 0.3365 | 0.3512 |
| Petroleum Ether % | 1.000 | 0.916 | 0.875 | 0.913 |
| Chloroform | 0.372 | 0.3644 | 0.3415 | 0.3673 |
| Chloroform | 0.4572 | 0.3807 | 0.3196 | 0.2938 |
| Chloroform Avg. | 0.4146 | 0.37255 | 0.33055 | 0.33055 |
| Chloroform % | 1.000 | 0.899 | 0.797 | 0.797 |
| Ethyl Acetate | 0.4495 | 0.3773 | 0.3306 | 0.3195 |
| Ethyl Acetate | 0.4543 | 0.3648 | 0.3716 | 0.3361 |
| Ethyl Acetate Avg. | 0.4519 | 0.37105 | 0.3511 | 0.3278 |
| Ethyl Acetate % | 1.000 | 0.821 | 0.777 | 0.725 |
| Water + IPA Mix | 0.575 | 0.4166 | 0.3868 | 0.385 |
| Water + IPA Mix | 0.4649 | 0.4217 | 0.4127 | 0.3761 |
| Water + IPA Mix Avg. | 0.51995 | 0.41915 | 0.39975 | 0.38055 |
| Water + IPA Mix % | 1.000 | 0.806 | 0.769 | 0.732 |
| CTH DA Oil | 0.5593 | 0.382 | 0.3392 | 0.3347 |
| CTH DA Oil | 0.5448 | 0.3602 | 0.3435 | 0.3448 |
| CTH DA Oil Avg. | 0.55205 | 0.3711 | 0.34135 | 0.33975 |
| CTH DA Oil % | 1.000 | 0.672 | 0.618 | 0.615 |

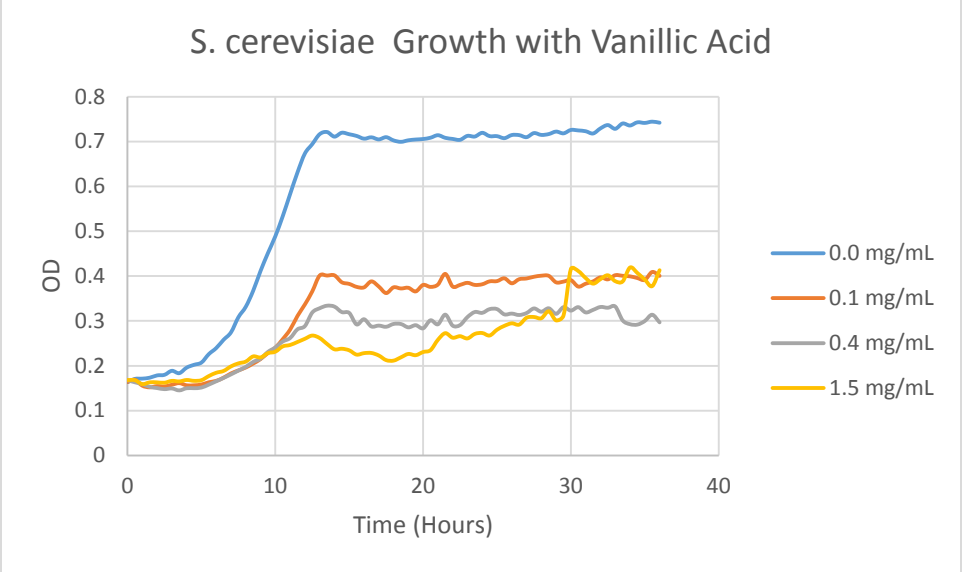
*E.coli* incubation with CTH AL fractionations

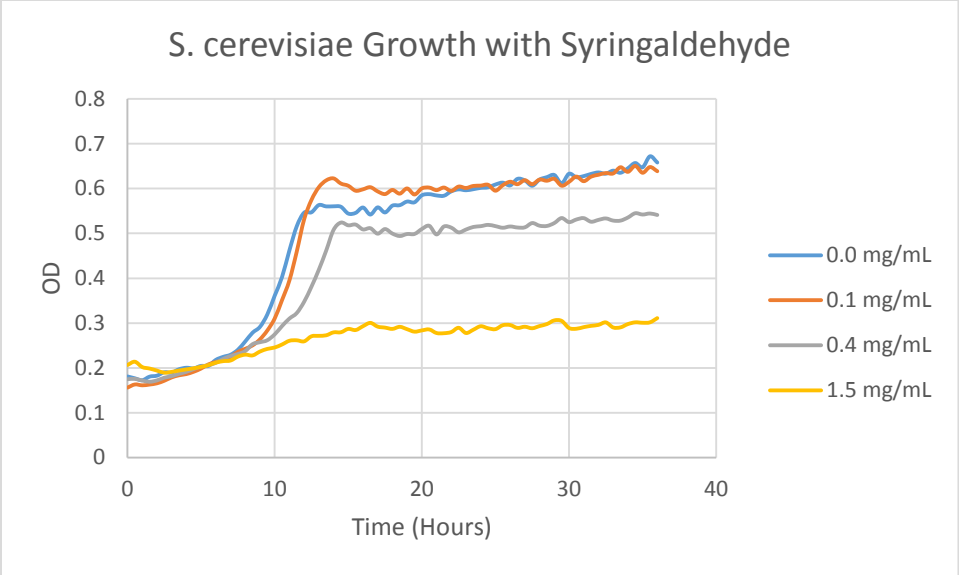
| | 0 mg/ml | 0.1 mg/ml | 0.4 mg/ml | 1.5 mg/ml |
|---|---|---|---|---|
| Hexane | 0.3123 | 0.2494 | 0.3449 | 0.3481 |
| Hexane | 0.3706 | 0.2978 | 0.2651 | 0.2726 |
| Hexane Avg. | 0.34145 | 0.2736 | 0.305 | 0.31035 |
| Hexane % | 1.000 | 0.801 | 0.893 | 0.909 |
| Petroleum Ether | 0.3221 | 0.3121 | 0.251 | 0.2866 |
| Petroleum Ether | 0.3323 | 0.2944 | 0.2507 | 0.2229 |
| Petroleum Ether Avg. | 0.3272 | 0.30325 | 0.25085 | 0.25475 |
| Petroleum Ether % | 1.000 | 0.927 | 0.767 | 0.779 |

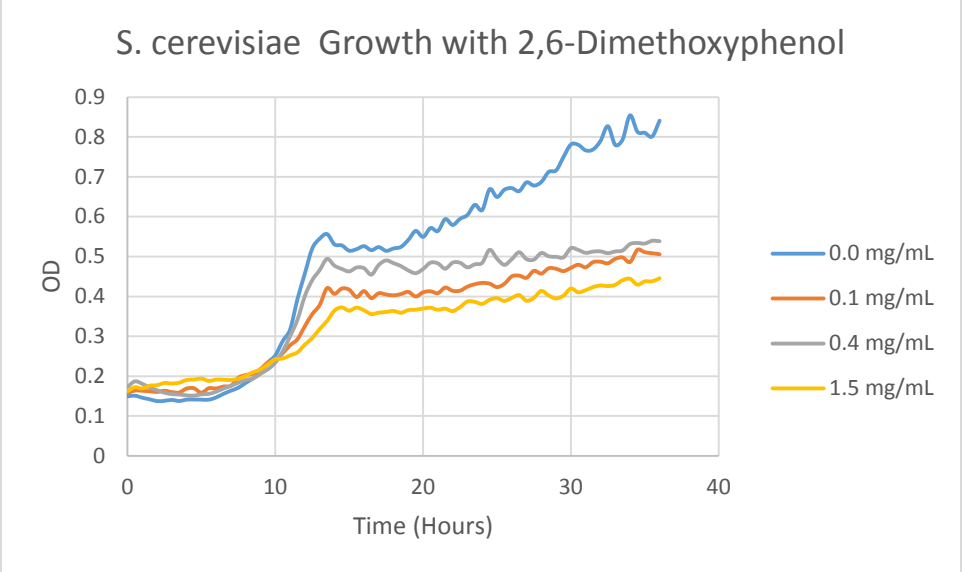
| | | | | |
|---|---|---|---|---|
| Chloroform | 0.3211 | 0.2785 | 0.2494 | 0.2591 |
| Chloroform | 0.3587 | 0.2915 | 0.2586 | 0.2362 |
| Chloroform Avg. | 0.3399 | 0.285 | 0.254 | 0.24765 |
| Chloroform % | 1.000 | 0.838 | 0.747 | 0.729 |
| Ethyl Acetate | 0.3156 | 0.3001 | 0.2655 | 0.2673 |
| Ethyl Acetate | 0.3384 | 0.3652 | 0.3193 | 0.3021 |
| Ethyl Acetate Avg. | 0.327 | 0.33265 | 0.2924 | 0.2847 |
| Ethyl Acetate % | 1.000 | 1.017 | 0.894 | 0.871 |
| Water + IPA Mix | 0.3447 | 0.2932 | 0.2907 | 0.2643 |
| Water + IPA Mix | 0.3798 | 0.3193 | 0.3239 | 0.3082 |
| Water + IPA Mix Avg. | 0.36225 | 0.30625 | 0.3073 | 0.28625 |
| Water + IPA Mix % | 1.000 | 0.845 | 0.848 | 0.790 |
| CTH DA Oil | 0.2847 | 0.2868 | 0.2864 | 0.3025 |
| CTH DA Oil | 0.3354 | 0.3036 | 0.3424 | 0.3146 |
| CTH DA Oil Avg. | 0.31005 | 0.2952 | 0.3144 | 0.30855 |
| CTH DA Oil % | 1.000 | 0.952 | 1.014 | 0.995 |

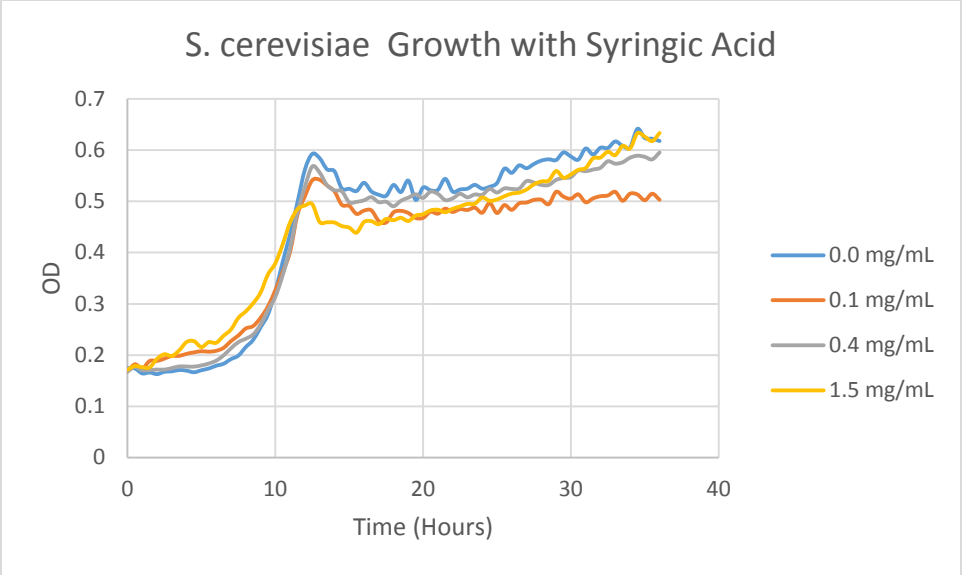
*Appendices G. Growth Curves of Microbes with Lignin Compounds*

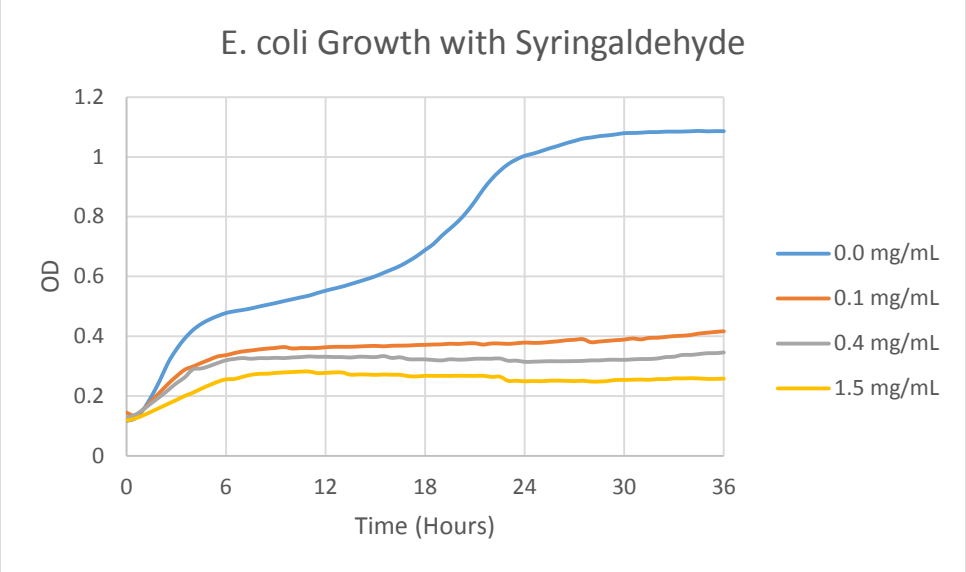


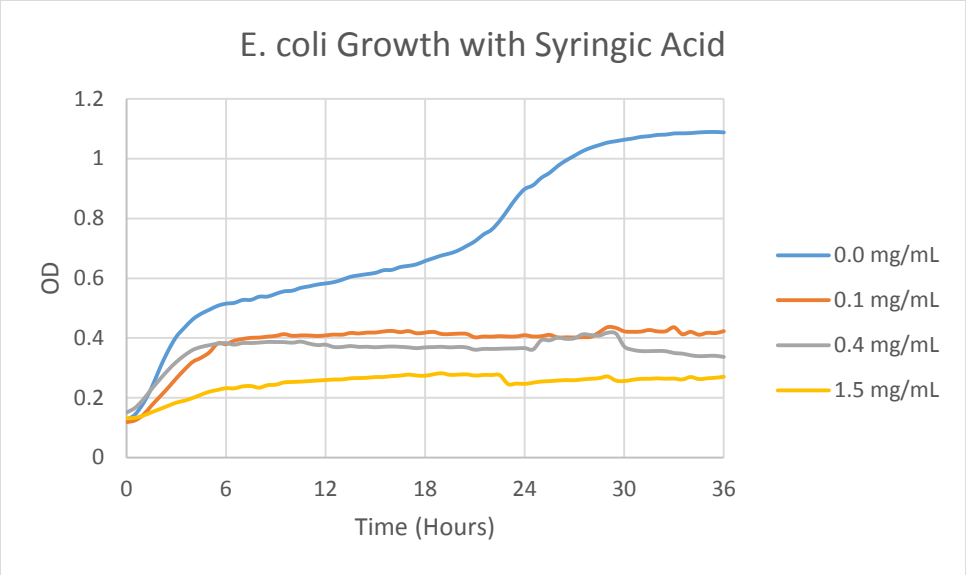



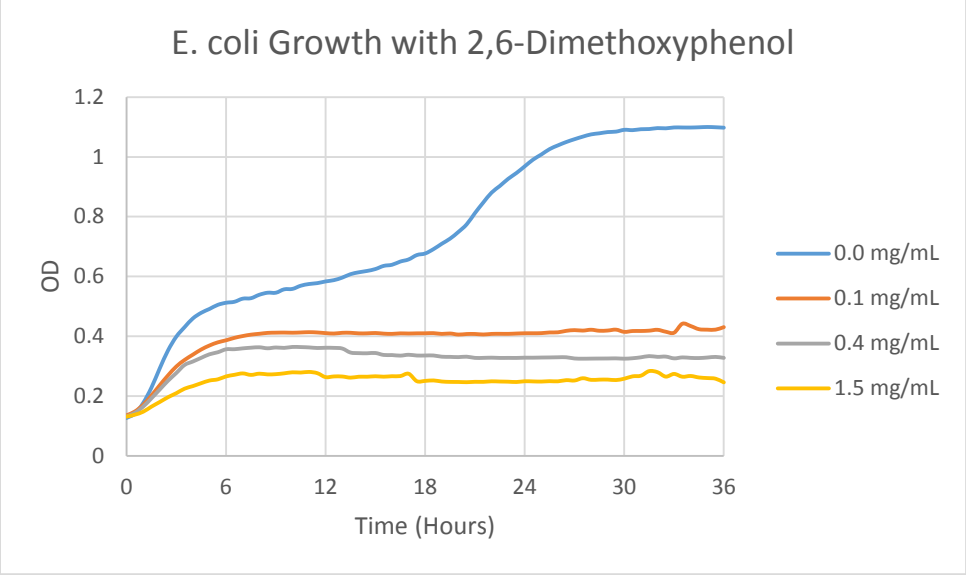

S. cerevisiae Growth with Syringaldehyde
OD
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL

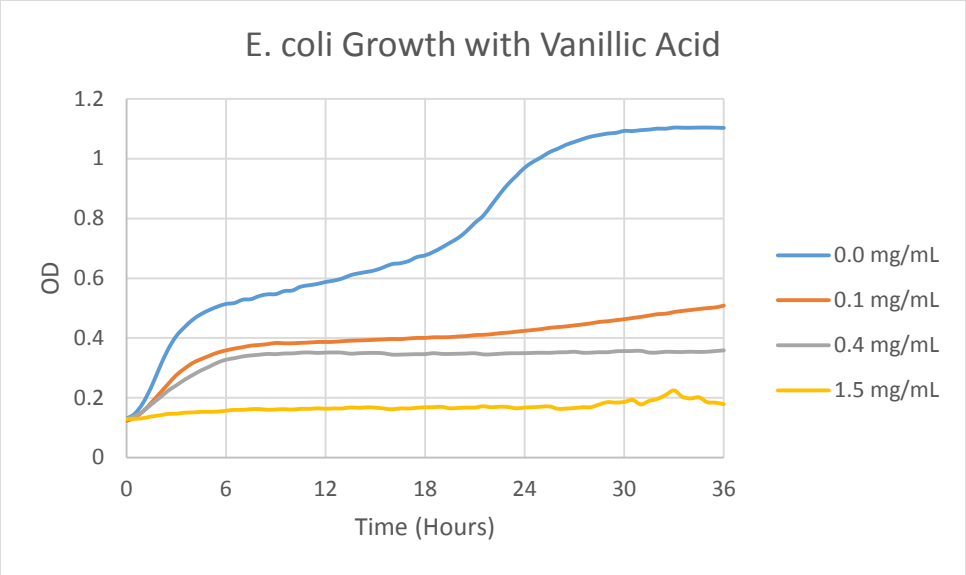

S. cerevisiae Growth with 2,6-Dimethoxyphenol
OD
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL

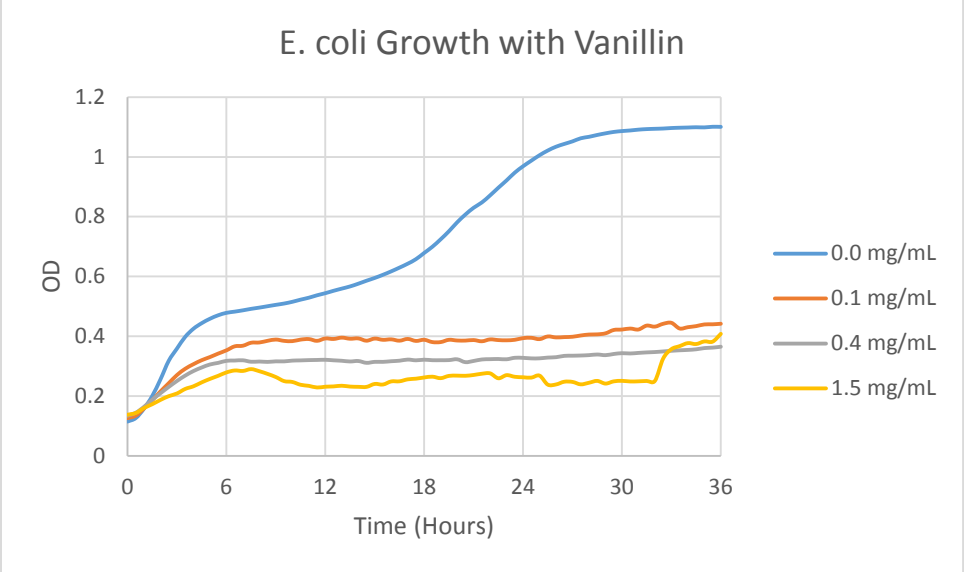

S. cerevisiae Growth with Syringic Acid
OD
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL

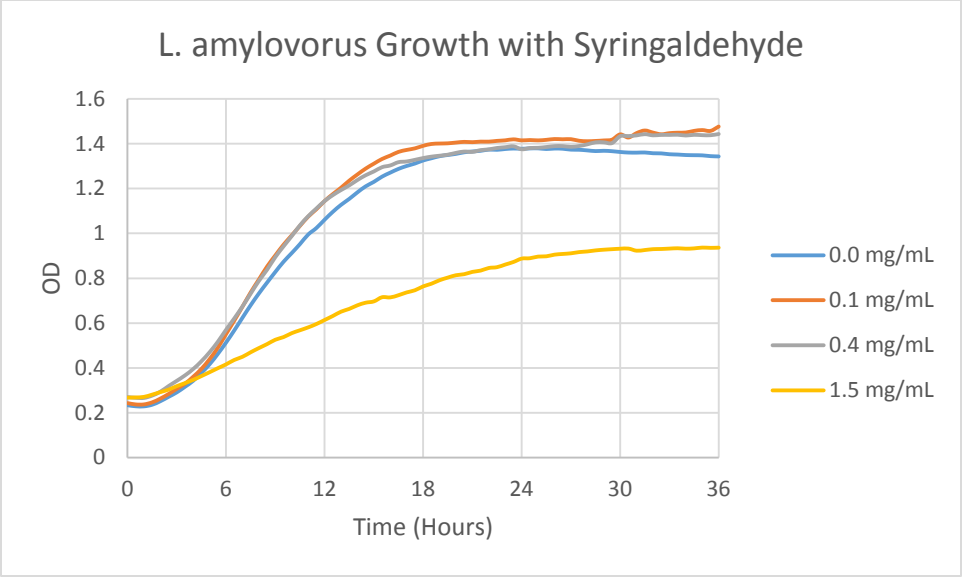

E. coli Growth with Syringaldehyde
OD
1.2
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL

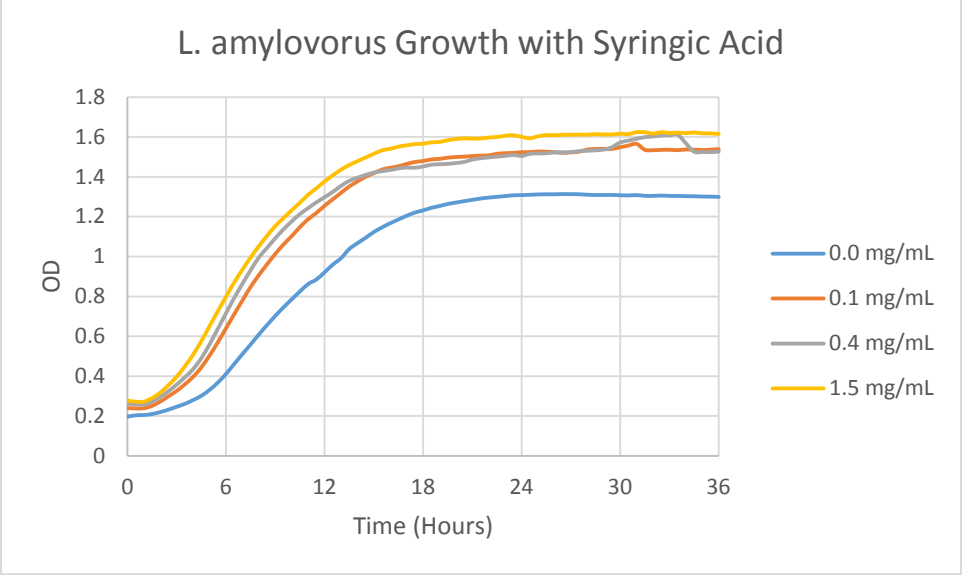

E. coli Growth with Syringic Acid
OD
1.2
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL

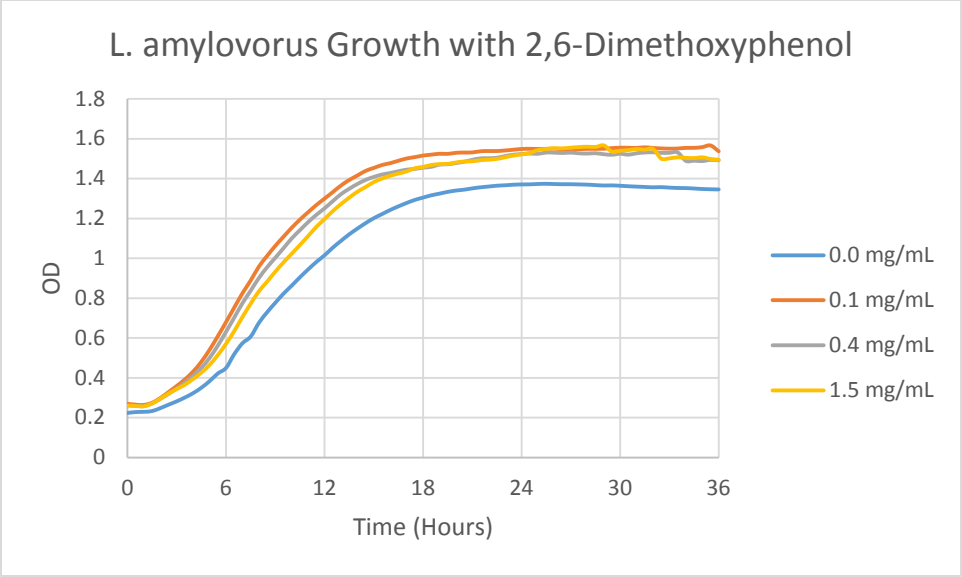

E. coli Growth with 2,6-Dimethoxyphenol
OD
1.2
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL

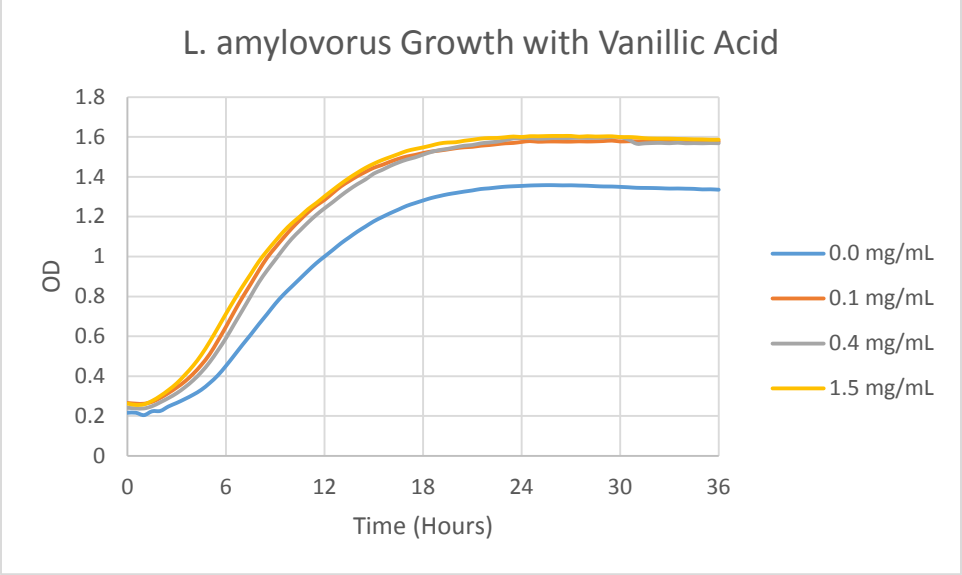

E. coli Growth with Vanillic Acid
OD
1.2
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL

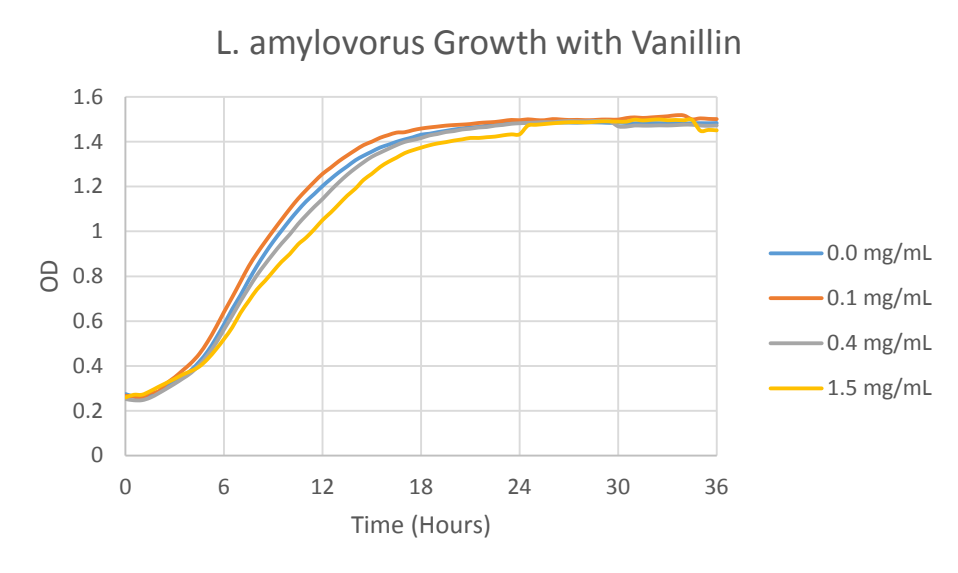

E. coli Growth with Vanillin
OD
1.2
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL


L. amylovorus Growth with Syringaldehyde
OD
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL

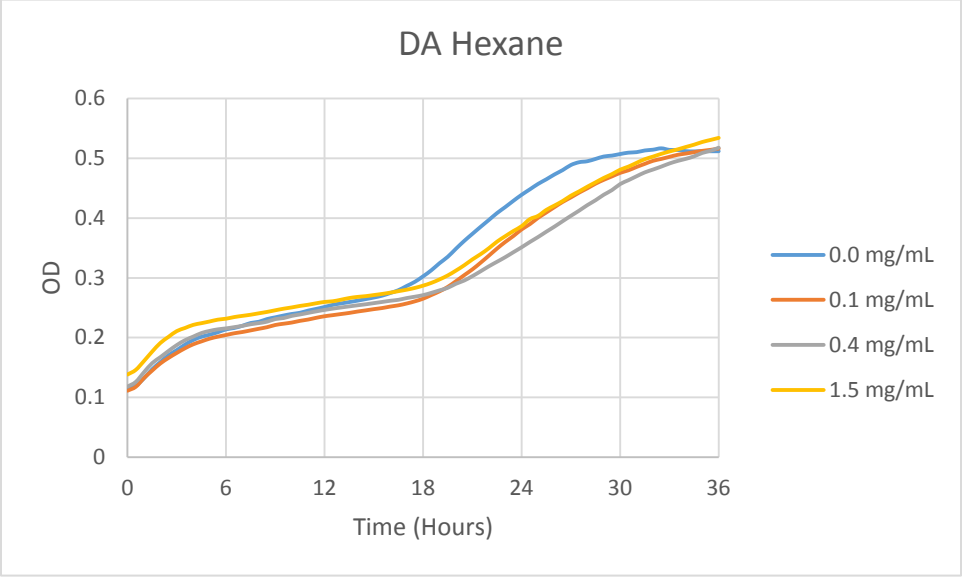

L. amylovorus Growth with Syringic Acid
OD
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL

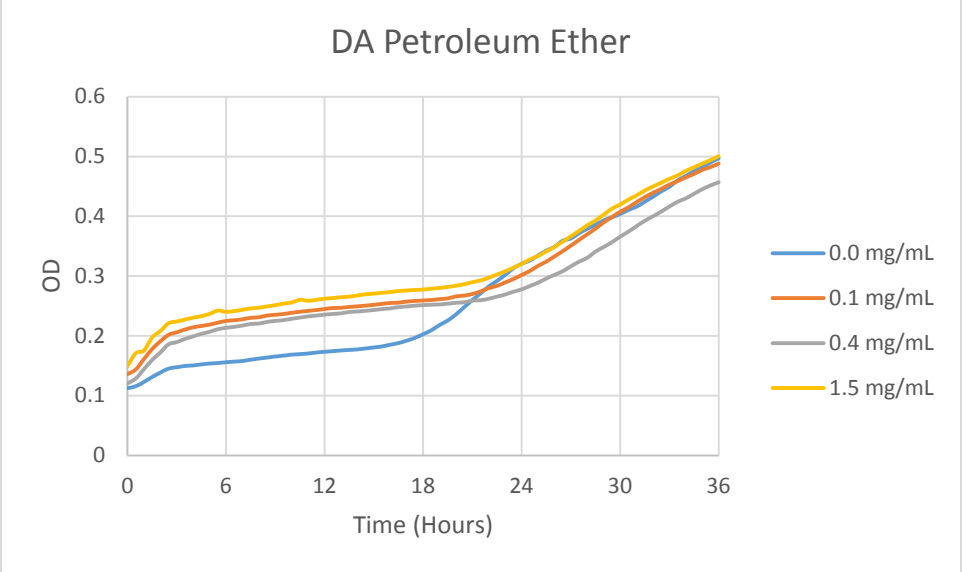

L. amylovorus Growth with 2,6-Dimethoxyphenol
OD
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL

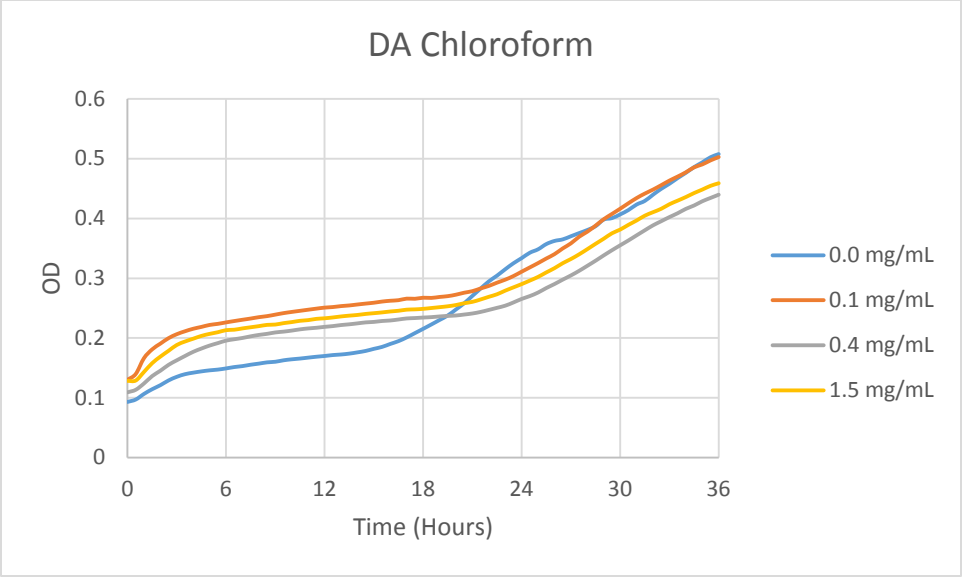

L. amylovorus Growth with Vanillic Acid
OD
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL

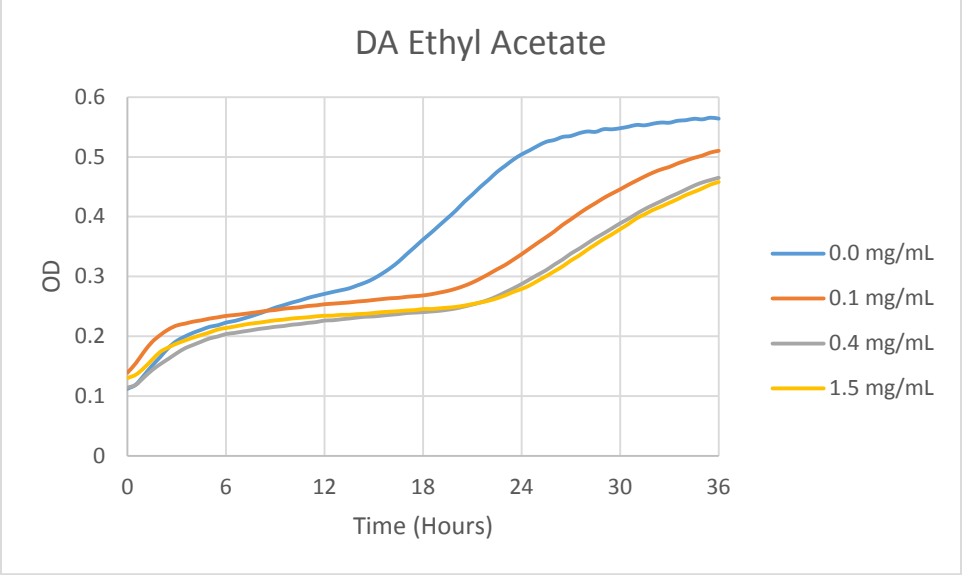

L. amylovorus Growth with Vanillin
OD
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL

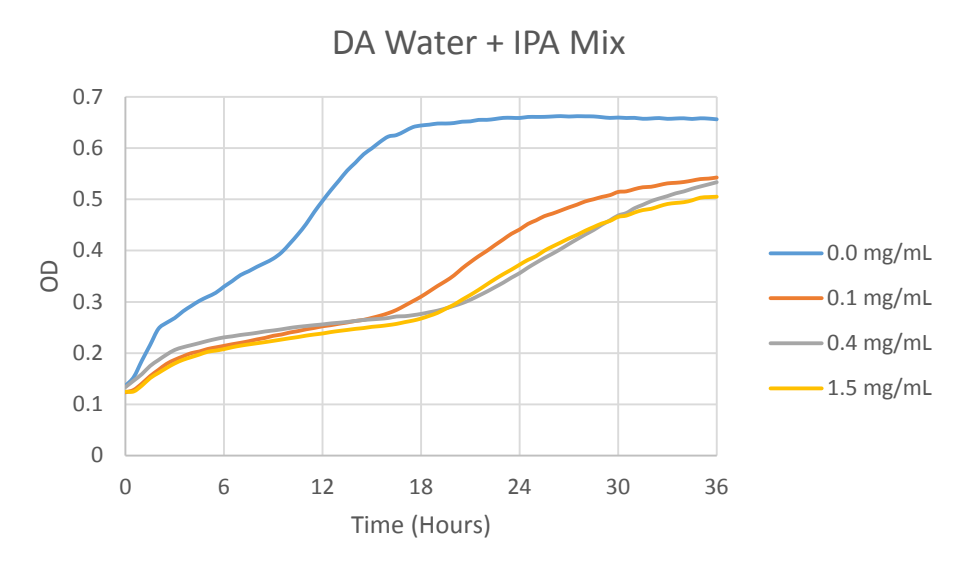
*Appendices H. Growth Curves of E. coli with CTH Lignin Oil and Fractionations*

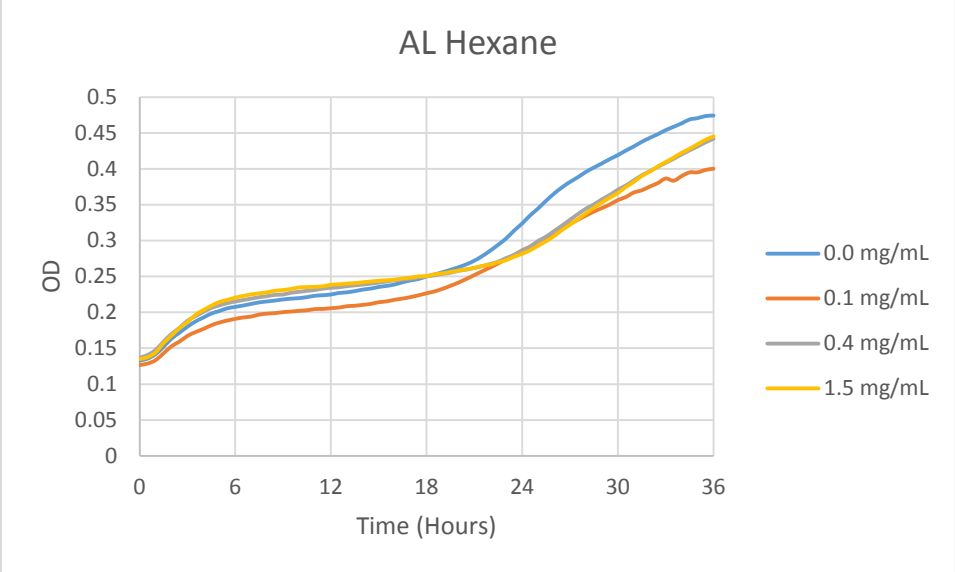


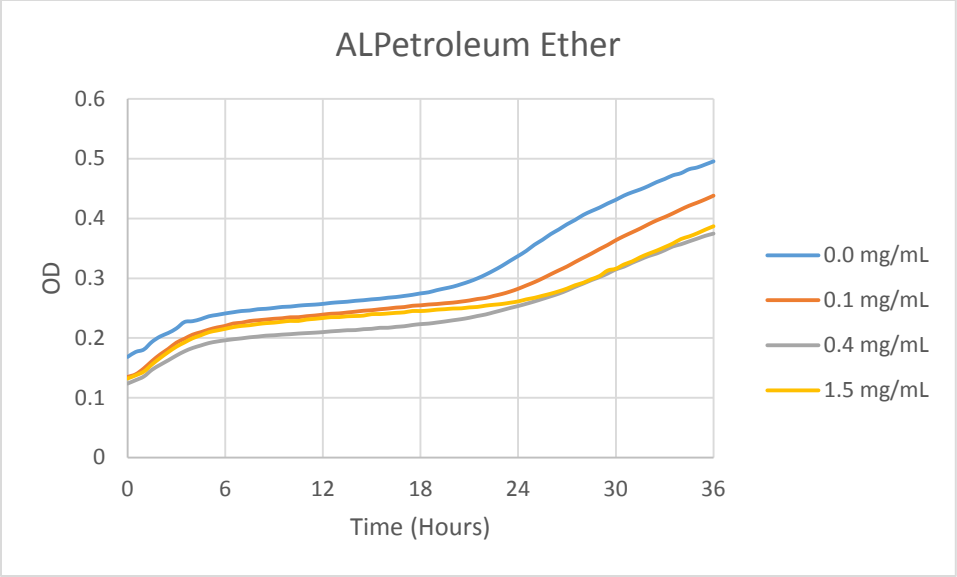



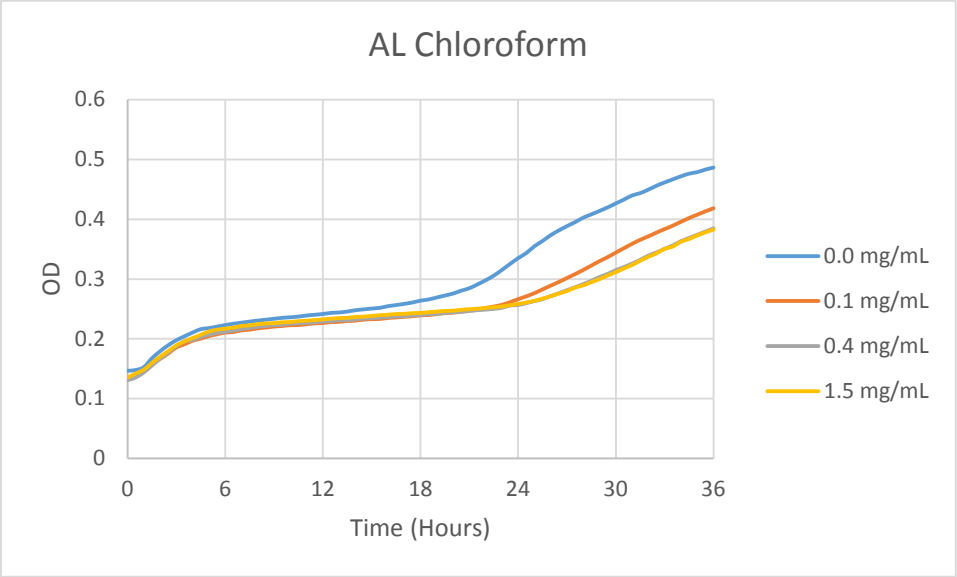

DA Chloroform
OD
0.6
0.5
0.4
0.3
0.2
0.1
0
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL

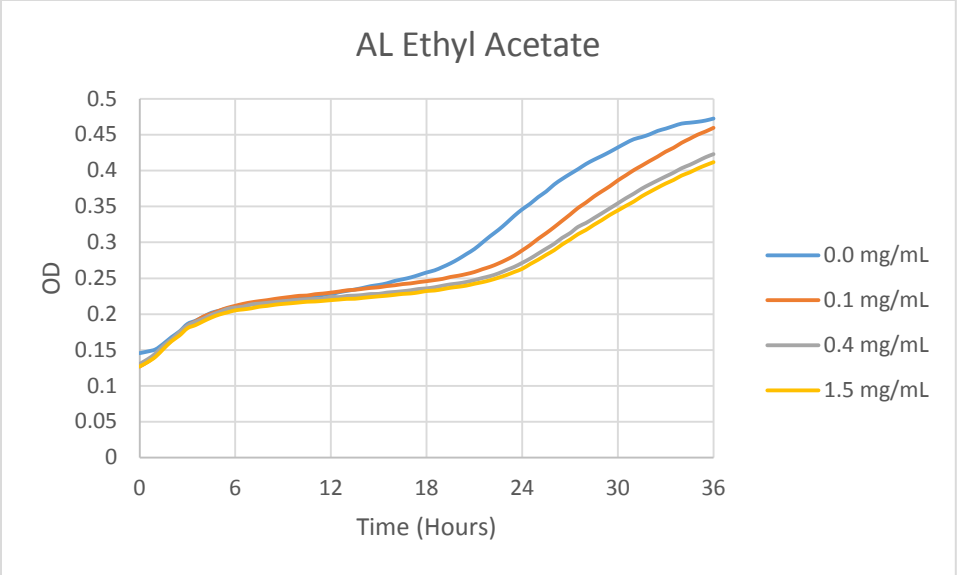

DA Ethyl Acetate
OD
0.6
0.5
0.4
0.3
0.2
0.1
0
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL

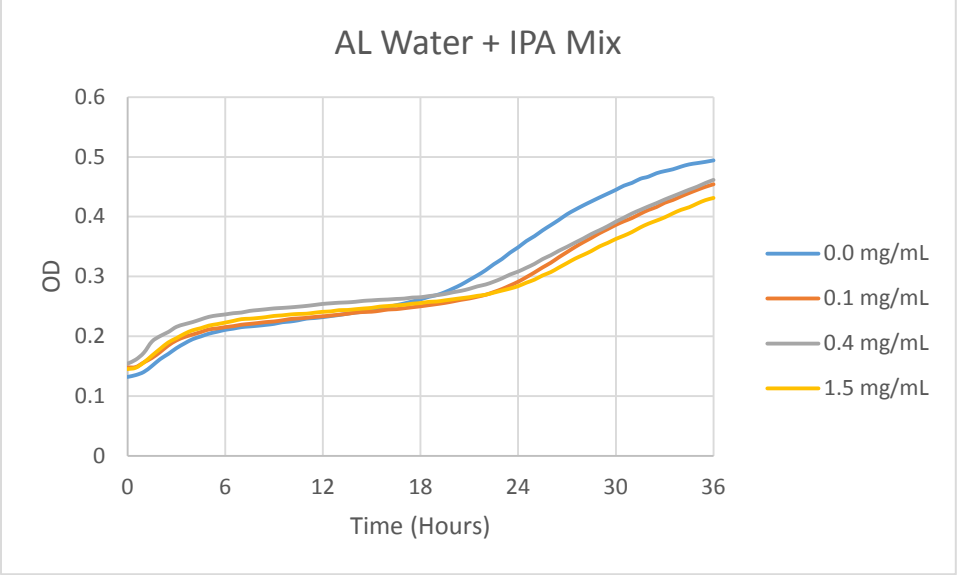

DA Water + IPA Mix
OD
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL


AL Hexane
OD
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL


ALPetroleum Ether
0.6
0.5
0.4
0.3
0.2
0.1
0
OD
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL


AL Chloroform
0.6
0.5
0.4
0.3
0.2
0.1
0
OD
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL


AL Ethyl Acetate
OD
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL


AL Water + IPA Mix
OD
0.6
0.5
0.4
0.3
0.2
0.1
0
0
6
12
18
24
30
36
Time (Hours)
0.0 mg/mL
0.1 mg/mL
0.4 mg/mL
1.5 mg/mL

SAS Code for Oils ANOVA

```
data OilYield;
input Block Treatments $ Oil;
datalines;
1 A 16.02
1 B 14.67
2 A 24.77
2 B 16.76
3 A 9.32
3 B 7.22
4 A 10.87
4 B 8.97
;
/* DA Lignin to AL Lignin*/
proc anova data=OilYield;
class Block Treatments;
model Oil=Block Treatments;
run;
```

SAS Output from Oils ANOVA

**The SAS System**

**The ANOVA Procedure**

| Class Level Information | | |
|---|---|---|
| Class | Levels | Values |
| Block | 4 | 1 2 3 4 |
| Treatments | 2 | A B |

| | |
|---|---|
| Number of Observations Read | 8 |
| Number of Observations Used | 8 |

**The SAS System**

**The ANOVA Procedure**

**Dependent Variable: Oil**

| Source | DF | Sum of Squares | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Model | 4 | 214.9733000 | 53.7433250 | 10.98 | 0.0389 |
| Error | 3 | 14.6901000 | 4.8967000 | | |
| Corrected Total | 7 | 229.6634000 | | | |

| R-Square | Coeff Var | Root MSE | Oil Mean |
|---|---|---|---|
| 0.936036 | 16.30091 | 2.212849 | 13.57500 |

| Source | DF | Anova SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Block | 3 | 192.6621000 | 64.2207000 | 13.12 | 0.0313 |
| Treatments | 1 | 22.3112000 | 22.3112000 | 4.56 | 0.1225 |

SAS Code for Oils T-test

```
data oil2;
input TRT $ Hydro_Acid_to_Alk;
datalines;
Acid  0.0932
Acid  0.0722
Alkaline    0.1087
Alkaline    0.0897
;
/* Hydro Acid to Alk... LDAS*/
proc ttest data=oil2
H0=0 SIDES=2;
class TRT;
var Hydro_Acid_to_Alk;
run;

data oil3;
input TRT $ Pyrolysis_Acid_to_Alkaline;
datalines;
Acid 0.1602
Acid 0.1467
Alkaline 0.2477
Alkaline 0.1676
;
/* Pyrolysis Acid to Alkaline */
proc ttest data=oil3
H0=0 SIDES=2;
class TRT;
var Pyrolysis_Acid_to_Alkaline;
run;

data oil4;
input TRT $ DA_Pyro_to_Hydro;
datalines;
pyro 0.1602
pyro 0.1467
CTH 0.0932
CTH 0.0722
;
/*DA Pyro to Hydro */
proc ttest data=oil4
H0=0 SIDES=2;
class TRT;
var DA_Pyro_to_Hydro;
run;
data oil5;
input TRT $ AL_Pyro_to_Hydro;
datalines;
pyro 0.2477
pyro 0.1676
CTH 0.1087
CTH 0.0897
;
/*AL Pyro to Hydro */
proc ttest data=oil5
H0=0 SIDES=2;
```

```
class TRT;
var AL_Pyro_to_Hydro;
run;
```

SAS Output from Oil T-test

**The SAS System**

**The TTEST Procedure**

**Variable: Hydro_Acid_to_Alk**

| TRT | N | Mean | Std Dev | Std Err | Minimum | Maximum |
|---|---|---|---|---|---|---|
| Acid | 2 | 0.0827 | 0.0148 | 0.0105 | 0.0722 | 0.0932 |
| Alkaline | 2 | 0.0992 | 0.0134 | 0.00950 | 0.0897 | 0.1087 |
| Diff (1-2) | | -0.0165 | 0.0142 | 0.0142 | | |

| TRT | Method | Mean | 95% CL Mean | | Std Dev | 95% CL Std Dev | |
|---|---|---|---|---|---|---|---|
| Acid | | 0.0827 | -0.0507 | 0.2161 | 0.0148 | 0.00662 | 0.4738 |
| Alkaline | | 0.0992 | -0.0215 | 0.2199 | 0.0134 | 0.00599 | 0.4287 |
| Diff (1-2) | Pooled | -0.0165 | -0.0774 | 0.0444 | 0.0142 | 0.00737 | 0.0890 |
| Diff (1-2) | Satterthwaite | -0.0165 | -0.0780 | 0.0450 | | | |

| Method | Variances | DF | t Value | Pr > \|t\| |
|---|---|---|---|---|
| Pooled | Equal | 2 | -1.17 | 0.3641 |
| Satterthwaite | Unequal | 1.9803 | -1.17 | 0.3651 |

| Equality of Variances | | | | |
|---|---|---|---|---|
| Method | Num DF | Den DF | F Value | Pr > F |
| Folded F | 1 | 1 | 1.22 | 0.9364 |

**The SAS System**

**The TTEST Procedure**

**Variable: Pyrolysis_Acid_to_Alkaline**

| TRT | N | Mean | Std Dev | Std Err | Minimum | Maximum |
|---|---|---|---|---|---|---|
| Acid | 2 | 0.1535 | 0.00955 | 0.00675 | 0.1467 | 0.1602 |
| Alkaline | 2 | 0.2077 | 0.0566 | 0.0401 | 0.1676 | 0.2477 |
| Diff (1-2) | | -0.0542 | 0.0406 | 0.0406 | | |

| TRT | Method | Mean | 95% CL Mean | | Std Dev | 95% CL Std Dev | |
|---|---|---|---|---|---|---|---|
| Acid | | 0.1535 | 0.0677 | 0.2392 | 0.00955 | 0.00426 | 0.3046 |
| Alkaline | | 0.2077 | -0.3012 | 0.7165 | 0.0566 | 0.0253 | 1.8074 |
| Diff (1-2) | Pooled | -0.0542 | -0.2290 | 0.1206 | 0.0406 | 0.0211 | 0.2553 |
| Diff (1-2) | Satterthwaite | -0.0542 | -0.5091 | 0.4007 | | | |

| Method | Variances | DF | t Value | Pr > \|t\| |
|---|---|---|---|---|
| Pooled | Equal | 2 | -1.33 | 0.3137 |
| Satterthwaite | Unequal | 1.0568 | -1.33 | 0.4005 |

| Equality of Variances | | | | |
|---|---|---|---|---|
| Method | Num DF | Den DF | F Value | Pr > F |
| Folded F | 1 | 1 | 35.20 | 0.2126 |

## The SAS System

### The TTEST Procedure

Variable: DA_Pyro_to_Hydro

| TRT | N | Mean | Std Dev | Std Err | Minimum | Maximum |
|---|---|---|---|---|---|---|
| CTH | 2 | 0.0827 | 0.0148 | 0.0105 | 0.0722 | 0.0932 |
| pyro | 2 | 0.1535 | 0.00955 | 0.00675 | 0.1467 | 0.1602 |
| Diff (1-2) | | -0.0708 | 0.0125 | 0.0125 | | |

| TRT | Method | Mean | 95% CL Mean | | Std Dev | 95% CL Std Dev | |
|---|---|---|---|---|---|---|---|
| CTH | | 0.0827 | -0.0507 | 0.2161 | 0.0148 | 0.00662 | 0.4738 |
| pyro | | 0.1535 | 0.0677 | 0.2392 | 0.00955 | 0.00426 | 0.3046 |
| Diff (1-2) | Pooled | -0.0708 | -0.1245 | -0.0170 | 0.0125 | 0.00650 | 0.0784 |
| Diff (1-2) | Satterthwaite | -0.0708 | -0.1344 | -0.00712 | | | |

| Method | Variances | DF | t Value | Pr > \|t\| |
|---|---|---|---|---|
| Pooled | Equal | 2 | -5.67 | 0.0297 |
| Satterthwaite | Unequal | 1.706 | -5.67 | 0.0420 |

| Equality of Variances | | | | |
|---|---|---|---|---|
| Method | Num DF | Den DF | F Value | Pr > F |
| Folded F | 1 | 1 | 2.42 | 0.7274 |

## The SAS System

### The TTEST Procedure

Variable: AL_Pyro_to_Hydro

| TRT | N | Mean | Std Dev | Std Err | Minimum | Maximum |
|---|---|---|---|---|---|---|
| CTH | 2 | 0.0992 | 0.0134 | 0.00950 | 0.0897 | 0.1087 |
| pyro | 2 | 0.2077 | 0.0566 | 0.0401 | 0.1676 | 0.2477 |
| Diff (1-2) | | -0.1085 | 0.0412 | 0.0412 | | |

| TRT | Method | Mean | 95% CL Mean | | Std Dev | 95% CL Std Dev | |
|---|---|---|---|---|---|---|---|
| CTH | | 0.0992 | -0.0215 | 0.2199 | 0.0134 | 0.00599 | 0.4287 |
| pyro | | 0.2077 | -0.3012 | 0.7165 | 0.0566 | 0.0253 | 1.8074 |
| Diff (1-2) | Pooled | -0.1085 | -0.2856 | 0.0687 | 0.0412 | 0.0214 | 0.2587 |
| Diff (1-2) | Satterthwaite | -0.1085 | -0.5219 | 0.3050 | | | |

| Method | Variances | DF | t Value | Pr > \|t\| |
|---|---|---|---|---|
| Pooled | Equal | 2 | -2.63 | 0.1189 |
| Satterthwaite | Unequal | 1.1122 | -2.63 | 0.2102 |

| Equality of Variances | | | | |
|---|---|---|---|---|
| Method | Num DF | Den DF | F Value | Pr > F |
| Folded F | 1 | 1 | 17.77 | 0.2965 |

SAS Code for Lignin, Glucan, and Xylan comparison

```
data comp;
input Lignin $ Lignin_Comparison;
datalines;
DA 61.55
DA 64.11
AL 54.90
AL 62.92
;
/* DA Lignin to AL Lignin*/
proc ttest data=comp
H0=0 SIDES=2;
class Lignin;
var Lignin_Comparison;
run;
/*DA Glucan to AL Glucan */
data comp;
input Glucan $ Glucan_Comparison;
datalines;
DA 27.37
DA 27.71
AL 17.44
AL 19.82
;
/* DA Lignin to AL Lignin*/
proc ttest data=comp
H0=0 SIDES=2;
class Glucan;
var Glucan_Comparison;
run;
/*DA Xylan to AL Xylan */
data comp;
input Xylan $ Xylan_Comparison;
datalines;
DA 6.02
DA 6.05
AL 7.08
AL 8.08
;
/* DA Lignin to AL Lignin*/
proc ttest data=comp
H0=0 SIDES=2;
class Xylan;
var Xylan_Comparison;
run;
```

SAS Output for Lignin, Glucan, and Xylan comparison

## The SAS System

### The TTEST Procedure

Variable: Lignin_Comparison

| Lignin | N | Mean | Std Dev | Std Err | Minimum | Maximum |
|---|---|---|---|---|---|---|
| AL | 2 | 58.9100 | 5.6710 | 4.0100 | 54.9000 | 62.9200 |
| DA | 2 | 62.8300 | 1.8102 | 1.2800 | 61.5500 | 64.1100 |
| Diff (1-2) | | -3.9200 | 4.2093 | 4.2093 | | |

| Lignin | Method | Mean | 95% CL Mean | | Std Dev | 95% CL Std Dev | |
|---|---|---|---|---|---|---|---|
| AL | | 58.9100 | 7.9581 | 109.9 | 5.6710 | 2.5301 | 181.0 |
| DA | | 62.8300 | 46.5661 | 79.0939 | 1.8102 | 0.8076 | 57.7636 |
| Diff (1-2) | Pooled | -3.9200 | -22.0313 | 14.1913 | 4.2093 | 2.1916 | 26.4546 |
| Diff (1-2) | Satterthwaite | -3.9200 | -40.2303 | 32.3903 | | | |

| Method | Variances | DF | t Value | Pr > \|t\| |
|---|---|---|---|---|
| Pooled | Equal | 2 | -0.93 | 0.4500 |
| Satterthwaite | Unequal | 1.2017 | -0.93 | 0.5007 |

| Equality of Variances | | | | |
|---|---|---|---|---|
| Method | Num DF | Den DF | F Value | Pr > F |
| Folded F | 1 | 1 | 9.81 | 0.3934 |

### The TTEST Procedure

Variable: Glucan_Comparison

| Glucan | N | Mean | Std Dev | Std Err | Minimum | Maximum |
|---|---|---|---|---|---|---|
| AL | 2 | 18.6300 | 1.6829 | 1.1900 | 17.4400 | 19.8200 |
| DA | 2 | 27.5400 | 0.2404 | 0.1700 | 27.3700 | 27.7100 |
| Diff (1-2) | | -8.9100 | 1.2021 | 1.2021 | | |

| Glucan | Method | Mean | 95% CL Mean | | Std Dev | 95% CL Std Dev | |
|---|---|---|---|---|---|---|---|
| AL | | 18.6300 | 3.5096 | 33.7504 | 1.6829 | 0.7508 | 53.7021 |
| DA | | 27.5400 | 25.3799 | 29.7001 | 0.2404 | 0.1073 | 7.6717 |
| Diff (1-2) | Pooled | -8.9100 | -14.0821 | -3.7379 | 1.2021 | 0.6259 | 7.5548 |
| Diff (1-2) | Satterthwaite | -8.9100 | -22.8392 | 5.0192 | | | |

| Method | Variances | DF | t Value | Pr > \|t\| |
|---|---|---|---|---|
| Pooled | Equal | 2 | -7.41 | 0.0177 |
| Satterthwaite | Unequal | 1.0408 | -7.41 | 0.0793 |

| Equality of Variances | | | | |
|---|---|---|---|---|
| Method | Num DF | Den DF | F Value | Pr > F |
| Folded F | 1 | 1 | 49.00 | 0.1807 |

## The SAS System

### The TTEST Procedure

Variable: Xylan_Comparison

| Xylan | N | Mean | Std Dev | Std Err | Minimum | Maximum |
|---|---|---|---|---|---|---|
| AL | 2 | 7.5800 | 0.7071 | 0.5000 | 7.0800 | 8.0800 |
| DA | 2 | 6.0350 | 0.0212 | 0.0150 | 6.0200 | 6.0500 |
| Diff (1-2) | | 1.5450 | 0.5002 | 0.5002 | | |

| Xylan | Method | Mean | 95% CL Mean | | Std Dev | 95% CL Std Dev | |
|---|---|---|---|---|---|---|---|
| AL | | 7.5800 | 1.2269 | 13.9331 | 0.7071 | 0.3155 | 22.5639 |
| DA | | 6.0350 | 5.8444 | 6.2256 | 0.0212 | 0.00946 | 0.6769 |
| Diff (1-2) | Pooled | 1.5450 | -0.6073 | 3.6973 | 0.5002 | 0.2604 | 3.1438 |
| Diff (1-2) | Satterthwaite | 1.5450 | -4.7840 | 7.8740 | | | |

| Method | Variances | DF | t Value | Pr > \|t\| |
|---|---|---|---|---|
| Pooled | Equal | 2 | 3.09 | 0.0908 |
| Satterthwaite | Unequal | 1.0018 | 3.09 | 0.1990 |

| Equality of Variances | | | | |
|---|---|---|---|---|
| Method | Num DF | Den DF | F Value | Pr > F |
| Folded F | 1 | 1 | 1111.11 | 0.0382 |

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